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CONTENTS OF VOL. 30, No. 3

The depletion of starch from the sapwood of the ash (*Fraxinus excelsior*) and its relation to attack by powder-post beetles (*Lyctus* spp.)

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With an appendix by E. W. BENNISON

The depletion of starch in the living sapwood of ash was examined as a possible means of rendering converted timber immune from attack by *Lyctus* (powder-post beetle).

Observations on disks of timber kept under controlled conditions showed that depletion is conditioned by access of oxygen; thus although in the standing tree depletion proceeds from without inwards, it can be induced in any part of the sapwood, and in any direction, by permitting access of oxygen, i.e. there is no polarity in depletion. The optimum temperature range for depletion in ash is from 31 to 36° C.: above 45° C. death of the cells may interrupt depletion.

The presence of β-indolyl acetic acid does not influence rate of depletion. Reformation of the starch in the depleted wood in the presence of cane sugar could not be induced. The enzyme concerned in mobilization of the starch appears to be a labile one with an optimum in the neighbourhood of 40° C. and to be produced during the active respiration of the cells, starch depletion ceasing when oxygen is withdrawn.

In transversely cut disks the rate of respiration at 33° C. ceases to be proportional to the volume of tissue after a thickness of about 6 mm. has been attained. At 20° C. disks 10 mm. thick may be evenly depleted. Infestation experiments upon timber undergoing depletion showed that the attack by *Lyctus* is circumscribed by starch-level and not by total nitrogen or soluble sugars.

Under correct conditions of kilning, 1 in. sapwood plank can be rendered starch-free in about 20 days: with larger sizes depletion is uncertain and probably uneconomic.

The methods of starch and sugar analysis used in the work are appended.

During recent years much attention has been focused upon the presence of starch in the sapwood of the large-pored hardwoods in its relation to the attack upon and the destruction of such sapwood by powder-post beetles (*Lyctus*). Mer's suggestion (1893) that wood starch is the food of the beetle was lost sight of until 1933 when Wilson recalled Mer's work and also demonstrated the progressive disappearance of starch along the gut of the beetle. Meanwhile, Campbell (1929) showed that in the powdery frass of the beetle the main cell-wall constituents are unchanged, and Clarke (1928) that in general the pore size of the vessels taken in relation to the size of the ovipositor of the beetle would circumscribe the attack to certain hardwoods. The seasonal removal and redeposition of starch in the standing tree was known, and Mer realized that if replacement of the starch mobilized in spring could be prevented before tree felling then a starch-free log would be obtained which should be immune from attack by *Lyctus*. He proposed several modes of procedure (Mer, 1903), including that of girdling the tree down to the cambium just below the crown, so that the flow of carbohydrates in the phloem would be interrupted and the replacement of starch prevented. His experiments were suggestive but not conclusive, and they were repeated and extended by Parkin (1938 *a*, *b*) and Parkin & Phillips (1939), whose work on girdled oak trees shows that as yet this method is insufficiently reliable for commercial production of *Lyctus*-immune timber.

The present experiments, begun in 1932 and carried on seasonally till 1939, were undertaken to examine the physiology of the disappearance of starch and to find whether timber already felled could be effectively 'destarched' on the commercial scale by physiological methods. A study of the drift of starch, nitrogen and sugars during the respiration of living wood during the period between felling and depletion was also made to find how far, if at all, the attack of *Lyctus* is limited by depletion of substances other than starch.

The depletion of starch. Once the timber is felled it becomes, physiologically, a community of dead and living cells with a capital of reserves which will gradually be consumed in normal katabolism up to the point of death. In theory, and assuming that starch-level is the limiting factor in attack by *Lyctus*, all that is necessary for the complete removal of starch is time, and in practice a log might either be kept intact and converted into plank after the starch had been respired away (the 'log-seasoning' of Wilson), or it might be first converted and then permitted to use up its starch. In either case it would be imperative that the water content of the timber should not fall below a level adequate for the continuation of life in the cells of the sapwood. Certain other factors, however, biological and economic,

must be considered. The fact that logs or planks are to be kept alive at a high moisture content involves the probability that bacterial action and fungal infection will occur, with degrade of the timber and consequent financial loss through stain and decay. Further, methods involving extra handling, spraying or other processing increase the cost of the finished product, perhaps beyond the economic limit. Finally, the consumer, if asked to pay extra for '*Lyctus*-immune' timber, requires some sort of guarantee that uniform destarching has taken place. With these considerations in mind the possible methods of destarching a sapwood may be briefly examined:

(1) *Girdling.* This, with its single initial outlay and simple technique, offers a cheap way of destarching if it can be perfected, but the treatment cannot yet be relied upon for the commercial production of *Lyctus*-immune timber (Parkin, 1938*b*). A certain measure of success can be achieved, but the occurrence of stain, decay, or irregular depletion preclude the use of the method.

(2) *Depletion in the log.* 'Log-seasoning', or the removal of starch by storage of the unconverted log, was noted by Mer (1903), and was advocated as a commercial possibility by Wilson (1933). The physiological necessity of maintaining a high water content for several years before normal seasoning to a low water content is permitted to take place invites loss through stain and decay (Parkin & Phillips, 1939). The depletion from logs immersed in water for a year or longer is more likely to yield clean, starch-free timber (Parkin, 1937).

(3) *Depletion in the plank.* The possibility remains of depleting timber already converted to plank, an aspect of the problem with which the present work is concerned, the basic hypothesis to be tested being that timber in the plank can be induced to consume its starch rapidly enough for commercial purposes.

For the experiments the sapwood of young ash saplings was employed, the trees being part of a stand at Knepp Castle, Horsham, Sussex. The trees, of which about 100 were used, were felled in the early morning and cut into small logs, of which two were selected from each tree, each being about 3 ft. long and 6 in. diam., with about 25 rings, all sapwood. The ends of the logs were at once sealed with gummed paper, and they were taken by car to the laboratory, where they were cut into the appropriate sizes, usually within 48 hr. of felling. For the depletion experiments a model seasoning kiln controlled to $\pm 1^\circ$ C. was used, and in all cases temperature and humidity were such as to ensure the continued life of the cells. Experiments were usually repeated about a dozen times, or until there was no doubt of the consistency of the results.

The disappearance of starch. In winter, in practically every parenchymatous cell of the wide sapwood of the young ash tree, i.e. right through to the pith in the 20–25-year-old sapling, copious reserves of starch occur in the form of grains. In the spring mobilization of this starch begins from the outer layers against the cambium and may proceed to complete or only partial depletion towards the interior.

Tests were first made to compare the march of oxygen uptake of this respiring sapwood with the disappearance of the starch. Small cubes of 1 cm. side were put into a simple microrespirometer immersed in a thermostat. The cube of wood was normally in communication with the outer air, except for 1 hr. periods once or twice a day, when the manometer connexion was made and the rate of oxygen uptake measured. At the same time the disappearance of starch was observed in similar cubes, one of which was used each time for a starch test with iodine. Respiration data obtained in this way are relative and not comparable from tree to tree, as they depend upon the differing proportion of living cells in the wood. It was hoped that there might in each case be a period of comparatively constant respiration rate until the starch in the living cells and the sugars derived from its mobilization were consumed, after which there would be a fairly sharp fall. However, after an initial rise in respiration caused, presumably, through wounding during the cutting of the cubes, the later fall in respiration rate, though fairly rapid, takes place over several days, after which the rate remains constant. The cells do not become depleted simultaneously, but the starch disappears from the medullary ray cells long before it does from those of the paratracheal parenchyma. This fact is interesting because oxygen has apparently much easier access to the paratracheal parenchyma than to the cells of the medullary ray. The former abut directly on the vessels, which are in this case in direct communication with the outer air (there are no tyloses in ash timber), while the latter are packed closely together without intercellular spaces and are much farther away from a direct oxygen supply than are the paratracheal cells. This prior disappearance of starch from the rays has constantly been observed in the course of the work. It appeared from these experiments that the average rate of uptake of oxygen per milligram of green wood per hour in 1 cm. cubes was of the order of 0·05 c.c. at temperatures near 20° C., and that this uptake fell during destarching to about 0·015 c.c. in a few days, a rate maintained for over a month until the death of the cells supervened. No clear-cut depletion point or drop in respiration rate could be expected, nor any specific time for even depletion. Thus relative respiration rate cannot be used as a criterion of starch disappearance.

At temperatures as low as 20° C. depletion took place more rapidly from the regions near the surface of disks of timber than from farther in. It was clear that diffusion rate plays an important part in determining the access of oxygen to, and the removal of CO_2 from, the respiring cells in the wood, and thus the rate of respiration and of starch depletion. As it would be necessary to secure uniformity of depletion

throughout specimen pieces for chemical analysis and infestation by *Lyctus*, experiments were made to find what thickness of disk could safely be used for destarching without fear of unevenness of depletion, i.e. at what thickness of disk, cut transversely from a log, the respiration rate per unit of weight would begin to fall. Accepting for this purpose that CO_2 output is proportional to respiration rate, disks varying from 0·3 to 2·4 cm. thick were cut from a fresh log and their CO_2 output measured by standard Pettenkofer apparatus, titrating baryta against oxalic acid. If diffusion were limiting, then with increase of thickness of disk or of temperature the respiration rate would sooner or later cease to be proportional to the volume of tissue and would tend to be proportional to its surface. In order that the results from each disk should be comparable, determinations on all the disks of one batch were made successively on the same day and with precautions as to uninterrupted uniformity of temperature and similarity of previous conditions. The results show that for temperatures of about 33° C. with young ash whose transverse surface is exposed as two sides of a disk the respiration falls rapidly after a thickness of about 6 mm. has been attained. Thus, to ensure even depletion throughout the wood it would be unwise to use disks thicker than this. For lower temperatures correspondingly thicker disks could be used; e.g. at 20° C. disks 1 cm. thick deplete evenly.

Empirical tests were made to determine the temperature at which starch depletion takes place most rapidly. This temperature should be either that at which respiration is at its optimum over the whole period of depletion or, if depletion should be dependent solely upon enzyme action, that at which the enzyme action is at its optimum. Where time enters as a factor a maximum rate of respiration could not be kept up for the period needed for depletion, and the premature death of the cells kept at a high temperature would leave unaltered starch, unless the enzyme involved continued to act after the death of the cells. Determination of the time-temperature relations of respiration was therefore not attempted, but instead direct tests were made to find at what temperature total depletion was fastest. For each experiment a log was cut up into disks 5 mm. thick (the ends of the log being as usual rejected), and the bark peeled off down to the cambium and replaced by vaseline. The bark was removed because with relative humidities of the order of 95% it tended to mould. At these high humidities moulds did not usually form upon the wood itself so long as it was alive and was not actually wetted. The disks thus prepared were supported on glass rods over a layer of water in a shallow tray, within a tent of muslin, the ends of the muslin dipping into the water in the tray, so that a very humid atmosphere was maintained around the disks. A tray carrying a number of disks was put into each of a series of incubators of different temperature, the range extending from 20 to 50° C. by intervals of approximately 5° C. A disk was removed daily from each tray, split along a diameter, and the exposed radial surface tested with iodine, so that the march of depletion could be followed. The transverse surfaces of the disks cannot be tested, as the surface is composed of cells killed by the friction of the saw, and therefore permanently full of starch.

The results of one of a series of eight experiments are shown in Table 1.

The temperature range for the most rapid removal of starch is between 31 and 36° C. At lower temperatures the rate of depletion is less, as would be expected; at higher temperatures it is less, presumably because the optimum has been passed and the maximum has not been maintained. In the case under consideration, death and arrest of enzyme action took place at 52° C. before any visible depletion occurred. At 40° C. depletion was complete though more slowly than at 31 and 36° C. The irregular depletion at 47° C. was probably due to limiting diffusion. The experiments as a whole show that the optimum temperature for the removal of starch lies between 30 and 36° C., although the cells can survive at 40° C. for a considerable time.

TABLE 1

4 Sept. 1935, log felled at Horsham. 5 Sept., log cut into 5 mm. disks. 8 or 9 disks in each oven.

Date (Sept.)	21° C.	27° C.	31° C.	36° C.	40° C.	47° C.	52° C.
6	++	++	++	++	++	++	++
7	++	++	++	++	++	++	++
9	++	++	++	++	++	+	++
11	++	++	+−	+	++	++	++
13	++	+	−	−	+	+	++
					at centre		
14	++	+−	−	−	+	++	++
16	+−	−	−	−	+−	++	++
17	−	n.s.	−	−	+−	+−	n.s.
18	−	−	−	−	−	−	++

Starch estimated by eye as: ++ much; + fair; +− slight; − none; n.s. = no sample.

Differential rate of depletion. Depletion in the standing tree begins in spring at the periphery of the xylem and proceeds inwards along the rays, but much more slowly than in disks of wood. This would appear to be connected with the relations of gaseous exchange of the timber; yet the anomalous behaviour of the starch grains in the paratracheal parenchyma raises the question whether simple access of oxygen is the activating condition for the depletion of starch. Observations were made to test this. Disks of wood 1 cm. thick were vaselined on both transverse faces, the bark being left untouched. The controls were not vaselined and lost their starch in the expected manner: the vaselined disks had by that time apparently lost none. Access of oxygen or lowering of CO_2 concentration might therefore be responsible

for the depletion. To discover which, unvaselined disks were divided into segments numbered 1–24. The odd-numbered segments were kept in jars in a continually renewed atmosphere of nitrogen, the even-numbered controls being in similar jars normally aerated. In a typical experiment, after 13 days at 25° C. the controls were clear of starch, while those in the nitrogen were unchanged. That they were still alive was shown by the fact that when transferred to the normally aerated jars they also lost their starch, in this case after a further 16 days. Similar results were obtained in seven trials out of eight. As CO_2 was free to diffuse away in both cases, the essential condition for depletion was concluded to be access of oxygen.

The question of polarity of depletion was examined to ascertain whether depletion in any part of the disk, with ample oxygen supply, takes place as it does in the standing tree—from the periphery inwards. Disks were taken and the outer three or four rings, which usually contain little or no starch, were removed. The remainder of each disk was vaselined on the transverse faces, and divided into narrow segments, whose radial faces were then vaselined. Each segment was divided tangentially along a ring to leave exposed two tangential surfaces—one facing outwards, the other inwards, all other surfaces being vaselined. The march of depletion was then observed upon samples withdrawn daily. Depletion occurred to the same extent from all the tangential faces exposed: from vaselined faces there was no depletion. Thus with isolated pieces of living sapwood depletion is not unidirectional.

Although oxygen thus appears to be the controlling factor, tests were made to discover whether cambium and phloem exert any influence upon depletion. Disks 70 mm. thick were taken, two longitudinal cuts on the exterior were made diametrically opposite each other, and the bark, phloem and cambium removed from one-half of the circumference. In some disks the whole tangential surface (half bark, half exposed sapwood) was vaselined: in others no vaseline was applied. The vaselined disks showed no depletion, while in those left unvaselined depletion occurred much more rapidly inwards from the exposed sapwood than from the portion still covered with bark. This confirms the fact that access of oxygen conditions depletion, and also shows that there is apparently no substance diffusing across from the region of the phloem which activates or favours the activation of the enzyme in the cells of the sapwood. In these experiments the possibility of depletion in the peeled portion being caused by some wound reaction is ruled out by the behaviour of the vaselined controls.

Certain experiments were made to ascertain whether a growth-promoting substance (β-indolyl acetic acid) would increase the amount of depletion, as such a substance might conceivably be present in the ascending sap in spring. Segments of disks were either stood upon their radial edges upon filter paper kept moist with a solution of the hormone (1/10,000) renewed twice a day, or had the radial surface smeared with a paste of the hormone in lanoline (1/5000). No difference appeared in the amount of depletion, although a little sporadic callus was formed upon the surface of those segments treated with the aqueous solution. Experiments were carried out upon disks, depleted but alive, to discover whether it is possible to induce reformation of starch. The disks were placed in cane-sugar solutions of various strengths up to 1 mol. and the controls in water, and were kept for periods up to about a month, or, in some cases, until fermentation supervened. No reappearance of starch was induced.

Finally, with commercial depletion of starch in the plank in view, two series of tests were made to determine the relative rates of march of depletion along and across the wood. In the first series a number of 1 in. cubes of living sapwood were taken and four faces, e.g. both radial and both tangential, were vaselined. The cubes were kept under the usual conditions of warmth and moisture, and samples taken daily. The cubes whose two transverse faces were left uncovered became depleted in about 10 days at 20° C., and much more rapidly than those whose radial or tangential faces had been left clear. These took about 3 weeks, no constant difference being observed between radial and tangential rates. In commercial practice, where planks are either quarter-sawn (radially) or plain-sawn (tangentially), a difference between these rates might be important, so confirmatory tests were made upon a number of sticks about 2 ft. long and of 1 in. square transverse section, cut with the annual rings parallel to one face, and having the ends and one pair of opposite faces, either the radial or the tangential, vaselined. Diffusion was thus permitted only across the tangential or the radial faces, and the conditions within the wood simulated those in the body of the full-size plank. No appreciable difference in the rate of depletion was observed; in commercial practice, therefore, time of depletion would depend little upon how the timber was sawn.

A few observations were made upon the enzyme responsible for the disappearance of the starch. They need amplification and verification, but the indications are that, like diastase, the enzyme has a high optimum at about 45–50° C., but unlike diastase, it appears to break down or to be inactivated after about 24 hr.

Starch depletion and infestation by Lyctus. Parkin (1936) showed that while starch is necessary to the diet of *Lyctus*, a substance or substances soluble from the wood in water at 60° C. is also necessary in oak sapwood for normal larval development. Also, he was able to grow larvae of *Lyctus* upon a diet containing protein, sugar and starch, but free of wood, and to show that fats are not essential for the growth of larvae. In the past it has been assumed

that starch content is the controlling factor in the development of *Lyctus*, the practical test being to expose a clean transverse surface of the timber, to stain with iodine, and to judge the timber immune if staining is slight or absent—a test which often proved deceptive. Moreover, soluble sugars up to 5·9% have been reported in sapwood (see Mansour & Mansour-bek, 1934). Experiments were carried out to discover by analysis at what level of starch depletion *Lyctus* fails to infest, and to confirm that starch and not a falling nitrogen content (having in mind the high nitrogen content of the beetle) or sugar content is the limiting factor. In three successive years a log was cut into disks, put into the kiln, and slowly and evenly destarched. Daily random samples of the disks were taken, each one being split into three segments, one very narrow for the iodine test, the other two equal and each comprising almost half the disk. These were rapidly oven-dried at 100° C. and used one for infestation and the other for chemical analysis. The infestations were carried out by Dr E. A. Parkin at the Forest Products Research Laboratory, Princes Risborough. The analyses were made at the Imperial College by Dr E. W. Bennison, and included the estimation of starch, total sugars and total nitrogen, the methods of analysis, details of which appear in the appendix, being developed by him as part of a general scheme applicable to problems of timber technology. The results of one of the infestation experiments are shown in Table 2.

TABLE 2

8 Feb. 1937. Ash from Horsham, 25 rings. Felled 5 Feb. 1937, cut into 8 mm. disks and put into kiln 8 Feb. 1937. Water content (on dry weight) 45%. Kiln temperature 25° C. Humidity *c.* 90%.

Days in kiln	No. of samples	No. infested	Starch as % oven-dry wt.	Sugars as % oven-dry wt.	Total N % oven-dry wt.	Starch by iodine test
1	3	2	2·32	1·05	0·11	Abundant
3	3	3	2·11	0·86		Fair
4	3	1	1·70	0·90		Visibly less
5	3	0	1·54	0·83		Visibly less
6	3	0	0·91	0·88	0·11	Visibly less
7	3	0	0·47	0·86		Visibly less
8	3	0	0·31	0·73		None in rays
9	3	0	0·24	0·79		—
10	3	0	Trace	0·71	0·11	All gone

These figures, which agree in substance with those of the other two infestation experiments, show that the percentage of nitrogen does not alter, whilst that of the total sugars falls about 15% during the first 3 days and then remains constant till the seventh day, after which it slowly falls. The starch content falls rapidly and consistently from the first. There is no infestation below a starch content of 1·7%. This is surprising, as starch of this amount is fairly visible under a hand-lens, although it is very plainly reduced in the cells of the rays. In the other two experiments no infestation was found in timber with a starch content of less than 1·5% except for a doubtful infestation in one sample at 0·98%. There is little doubt that the lower limit of infestation is about 1·5%.

For the other two experiments the nitrogen percentage was 0·11 and 0·12. The respective sugar values were: 1·19%, falling to 0·85%, and 1·51%, falling to 0·89%.

Discussion. While the physiological experiments were simple tests to provide pointers for approach to the technological problem, yet they provide material for interesting speculation upon conditions within the tree. Disappearance of starch in the standing tree begins at the cambium and proceeds towards the centre. On bringing a log, whose ends are sealed, from winter conditions into a temperature of 25–30° C., depletion begins in the two or three outer rings, and this depletion always occurs whether oxygen is admitted or not. Depletion within the disk as a whole is not polar, and in a disk of sapwood whose faces are all vaselined, or in a sealed-up log, no depletion takes place except in the two or three most recent rings. This has constantly been noticed, and even in ash felled in midwinter the outer rings often contain no starch. But if starch is present in these rings its disappearance during the winter, or in the disk, is independent of transpiration current or of the access of oxygen in appreciable quantity. Also, it is not caused by the diffusion of substances across the cambium. The only reasonable explanation at present is that as these rings contain the most active conducting elements depletion in this region is due either to small residual quantities of oxygen or of enzyme remaining from the activity of the previous autumn. Depletion of the starch in the remainder of the sapwood can be caused at any time by the admission of oxygen. This, and the fact that depletion does not occur simultaneously throughout the wood, and often may not occur at all in the standing tree, rules out the possibility of any 'ripening' or dormancy period as a condition of mobilization. The evidence appears to show that it takes place through the action of an enzyme which is labile, and which can be produced by the katabolism of the cells irrespective of their distance from the cortical tissues. The common presence of a few rings of depleted wood next the cambium and the progressive disappearance of starch inwards as the season progresses suggest that the immediate cause of conversion of the starch has its origin in the outer part of the tree, and that inward diffusion occurs along the path of the rays. As the enzyme can be produced locally, and as there is no reason to suppose that an activator is produced in or is transported to the outer part of the sapwood, it seems more likely that in the standing tree as in the isolated disk oxygen alone is the regulating factor, and that it is

its cell-to-cell movement by diffusion and intracellular transport that permits the resumption of activity in the cells of the outer sapwood. The source of this oxygen is not the air outside the trunk, because the cortex acts as a barrier as effective as a layer of vaseline. The oxygen must be brought up in the ascending sap, and must move inward along the rays. Very often the inner layers of sapwood are not depleted, and this may occur for a number of years in succession. The undepleted cells are presumably without oxygen during the period when they are not depleted, and must live anaerobically for indefinite lengths of time; but they do not die, since at the change from sapwood to heartwood all their starch disappears. How this occurs is not known. It may be that there is a gradual formation of heartwood through suicidal respiration by the cells of oxygen which is transported or diffuses in the heartwood itself, i.e. that the heartwood is increased from within; or it may be that starch is removed during metabolism in spring and is not replaced in the innermost layers, which will therefore later die of starvation. The common inclusion of large aggregates of starch-bearing cells within the heartwood, as in the 'internal sapwood' of oak, tends to support the latter view, as it is possible that these cells have never been depleted and have remained alive in an anaerobic condition till the death of the protoplasm through prolonged dormancy.

There remains the anomalous behaviour of the paratracheal parenchyma cells whose starch does not disappear until long after that of the cells of the medullary rays, although in a disk of wood the latter are certainly further from a supply of oxygen. If the starch did not disappear at all from these cells it could be assumed that they were either dead or did not elaborate any enzyme, as the medullary ray cells do. They do at length deplete, however, and presumably get their oxygen more slowly. It may be that there is a difference in the permeability of the walls to oxygen, although no difference of structure can be seen under the microscope.

The infestation experiments show that *Lyctus* will not infest timber whose starch content is below about 1·5% of the dry weight. At this concentration starch is still clearly visible with the iodine test, which has been used commercially for grading timber as either *Lyctus*-immune or not. A plank which shows no coloration with iodine ought therefore to be immune; but this has not proved so in practice with timber that has been seasoned in the log. Thus depletion, when brought about by present methods, must be uneven, and freedom from starch in the plane of the iodine test does not imply freedom elsewhere in the piece.

The enormous and world-wide annual damage caused by *Lyctus* infestation even after timber has temporarily been rendered free by kiln drying makes the removal of starch from the sapwood of infestable species an urgent commercial problem, particularly as the extra width obtained by inclusion of the sapwood enhances the value of the timber. The work described above would seem to offer a method for the depletion of plank up to about 1–1¼ in. thick, if kiln conditions are sufficiently controllable and the oncost of depletion is not great enough to offset the profit obtainable on 'certified' wood. As the outer cells do not die for a long time after the last of the starch has gone, the access of oxygen to the inner cells is not a matter of simple diffusion or transport, because much of it is removed and used en route by the still-living outer cells. The practical result of this is that whereas a plank some ⅜ in. thick will be depleted in a week or so, a 1 in. plank takes about 3 weeks, which is near the economic limit, while a 2 in. plank, apart from the risk of degrade through stain, would take too long to be commercially feasible. In commercial practice, also, the temperature and humidity of the kiln would need to be controlled skilfully. In a semi-commercial test carried out at Princes Risborough following the appearance of a preliminary note (Henderson, 1935), depletion did not occur throughout the planks of oak and ash, presumably because the water content of the timber fell about 20% during the period of the test. Growths of *Penicillium* occurred, showing that the humidity must at times have reached an unduly high level. Since then, Parkin & Phillips have successfully depleted plank up to 3 in. thick, working on a semi-commercial scale at about 40° C., without the death of the timber or the incidence of fungal attack (unpublished data). Their findings confirm the conclusion reached above, namely, that 1 in. plank can be successfully destarched. The 2 and 3 in. sizes took much longer, and even depletion, especially in the 3 in. plank, could not be assured. A point of interest was the appearance of an irregular physiological darkening of the treated ash timber. This has not been experienced in the writer's work, which was done upon younger wood and mostly at lower temperatures. Such discoloration would be regarded as a degrade in the trade.

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APPENDIX

By E. W. Bennison

The estimation of starch in wood. 4–6 g., depending on the starch content, of 60–90-mesh wood are accurately weighed into a Soxhlet extraction thimble, covered with a disk of filter paper to prevent loss by splashing and extracted with 78% alcohol for 12 hr. The wood is dried at room temperature overnight and is then quantitatively transferred to a 250 ml. wide-mouthed conical flask and heated on the water-bath under reflux with 100 ml. 95% alcohol containing 1 ml. conc. HCl for 40 min. The mixture is filtered on a hardened paper without suction, and the wood washed completely free from acid with hot 95% alcohol, 400–500 ml. being necessary for this purpose.

After the alcohol has been well drained off, the wood is washed quantitatively with about 400 ml. of water into a 600 ml. beaker. The mixture is then boiled with mechanical stirring for 45 min. during which period the volume is reduced to about 150 ml. It is then cooled and made up to 200 ml. in a standard flask, after which the wood-dust is filtered off on a fluted paper.

To 25 ml. of the filtrate are added 2 ml. of acetate buffer, *p*H 4·63, 2 ml. of β-amylase solution and 3 drops of toluene. The flask is plugged with wool and incubated at 25° C. for about 42 hr. A blank is carried out with killed enzyme. The maltose produced, and hence the starch originally present, is estimated by Hanes's modification of the Hagedorn-Jensen method. Details of this and of the method of preparing the enzyme are given by Hanes (1936). In order to calculate percentage starch to original oven-dry wood it is necessary to determine: (*a*) moisture on original wood, (*b*) material extracted by alcohol, (*c*) moisture on wood after alcohol extraction.

The estimation of sugars in wood. About 2 g. of the wood-dust (60–90 mesh) is accurately weighed into a Soxhlet thimble and covered with a disk of filter paper to prevent loss by splashing. It is extracted with ether overnight, the ether allowed to evaporate and the wood quantitatively transferred to a piece of muslin placed in the neck of a 250 ml. wide-mouthed conical flask. The wood-dust is tied up in the muslin and the whole extracted with 150 ml. of 78% alcohol by boiling under reflux for 3 hr. The muslin bag is necessary to prevent bumping, and it is also advisable to add a little porous pot.

The extract is filtered through no. 1 paper into a glass evaporating basin (diameter approximately 5 in.), the muslin bag being twice washed with hot 78% alcohol. The bag is then opened and the contents washed into the funnel with hot alcohol. The total amount of alcohol required for the washings should be 400–450 ml. The evaporating basin should not be allowed to become more than two-thirds full as the alcohol is liable to 'creep' up the sides, and it is therefore necessary to evaporate down during the washing process. The extract is ultimately evaporated down to about 10 ml. in the basin, when it is transferred to a 50 ml. beaker, the dish being washed out successively with 95% alcohol, 78% alcohol, and twice with water.

The liquid in the beaker is then slowly evaporated down without boiling, on a hot plate or water-bath to about 7 ml. It is cooled, 6 drops of basic lead acetate solution (saturated) are added, and the whole allowed to stand overnight.

The mixture is then filtered through a small fluted no. 1 paper, the first few drops being returned to the filter if necessary. The beaker should be rubbed down with a 'policeman' and the precipitate washed thoroughly with about 400 ml. of distilled water.

The filtrate is then evaporated down to about 25 ml. and the excess lead precipitated as sulphide with H_2S. In order to get a clean precipitation it is necessary to have the liquid at about 60° C. and to pass the H_2S under slight pressure with shaking.

The lead sulphide is filtered off and washed with about 250 ml. of water. The filtrate is evaporated down on a water-bath to a few ml. and finally made up to 25 ml. in a graduated flask.

An aliquot (5 ml.) of this solution is diluted to 15 ml. and the reducing power of 5 ml. of this determined by Hanes's modification of the Hagedorn-Jensen method.

A further 5 ml. aliquot of the original solution is heated in a 50 ml. flask with 5 ml. of $2N$ sulphuric acid under a small reflux condenser on a water-bath for 5 hr. After cooling and allowing the condenser to drain completely, the acid is neutralized with 5 ml. of $2N$ sodium hydroxide, and the reducing power determined on 5 ml. of the mixture.

The figures both before and after hydrolysis are given in terms of glucose.

Nitrogen. Nitrogen was estimated as total nitrogen by the standard Kjeldahl method.

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Studies in the physiology of the onion plant

I. An investigation of factors concerned in the flowering ('bolting') of onions grown from sets and its prevention

Part I. Production and storage of onion sets, and field results

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(With Plate 3 and 1 Text-figure)

The investigation concerns the effects of five factors upon the tendency of onion sets to bolt and the production and ripening of bulbs. The factors studied fall into three categories: (1) conditions under which seedlings were grown to produce sets, (2) conditions of storage of sets during dormancy, (3) conditions of growth during the second season. The first comprised sowing date of seed (day-length and temperature effects) and temperature of growth while forming sets; the second, high- and low-temperature storage for different periods and throughout the winter; the third, temperature and day length during the second season. Field trials and experiments with controlled environment were carried out. The main results of the former were as follows: Large sets were much more liable to bolting than small. Sets produced at high temperature under glass were practically free from bolting when grown on, irrespective of the storage conditions. Storage throughout the winter (22 weeks) at 30° C. practically eliminated bolting; 30° C. for the *first* 8 weeks reduced bolting but given for the *last* 8 weeks was much less effective. Cold storage (0° C.) throughout or for the *first* 8 weeks considerably reduced bolting, but when given for the *last* 8 weeks tended towards increase. High-temperature storage leads to loss in weight; the maximum loss in any treatment, 75%, was associated with 77% mortality in the field. This detrimental effect on survival is confined to sets produced at high temperature (which require no heat treatment as they have no tendency to bolt), while sets produced at lower temperatures show no high mortality during or after high-temperature storage. The reduction in bolting following high-temperature storage is not due to water loss by the sets. High temperature of storage throughout or for the last 8 weeks delayed ripening of the bulbs, and resulted in larger yields; no such effect was obtained by high-temperature storage during the first 8 weeks.

Onion sets are small bulbs, which are produced from seed in one season, stored dry through the winter, and are intended for planting out to give large bulbs in the following season. They are produced and used on a large scale in the U.S.A., but in this country were in the past imported (generally from central and southern Europe), and their use was in the main confined to amateur gardeners. Onion sets might well be successfully produced on a commercial scale in this country, and their use offers advantages, but not uncommonly they run to seed in the second season and then the bulbs are generally small and of little commercial value.

Previous work in the U.S.A. has shown that the incidence of bolting is affected by variety, size of set, storage temperature and temperature during the second season. In a series of field experiments Thompson & Smith (1938) employed sets of four varieties stored at 30, 32, 40, 50 and 60–70° F. and graded into three size classes. These showed that storage at 40° F. gave the highest incidence of bolting, with a progressive decrease at both higher and lower temperatures. The lowest incidence followed 60–70° F. storage, which, however, caused withering and loss in store. Larger sets gave more flowering than smaller, and var. Ebenezer gave much less bolting than the other varieties.

In greenhouse experiments the same authors (1938) investigated the effects upon flowering and bulbing, of temperature and photoperiod during the growth from set to mature onion. Sets of vars. Ebenezer and Red Wethersfield were planted in October, i.e. without the usual period of winter storage. They were grown at 50–60, 60–70 and 70–80° F. under normal winter day length and also with additional electric light 'from about sundown until 10 p.m.' In the normal day length bulbing did not take place at *any* temperature. In long days (15 hr.) the higher temperatures gave earlier bulbing and maturity. There was, in fact, a large interaction between temperature and day length with respect to bulbing and ripening such that short days inhibited bulbing and longer days enabled it to occur at medium or high temperatures only. Practically all the sets produced seed stalks at 50–60° F. in either long or short days, whereas very few inflorescences appeared at 60–70° F. in either day length and none at 70–80° F. Day length apparently had no effect on inflorescence production except in a single experiment with Ebenezer in 1934–5. In this, 40% of the plants at 60–70° F. produced inflorescences in short days and only 5% in the longer days. As usual, nearly all the sets at 50–60° F. produced inflorescences in either day length and none at 70–80° F.

The greenhouse experiments of Thompson & Smith appear open to criticism in that the normal winter storage period was omitted. In considering the effects of photoperiod and temperature upon flowering behaviour, it is important whether inhibition of flowering occurs at the stage of initiation of primordia or at one of the various later stages of development, as was stressed by Gregory (1936). Thompson & Smith (1938) state that in sets of Ebenezer stored at various temperatures through the winter, inflorescence primordia were not found before the end of March in large sets and 10 Apr. in medium-sized sets; the same was found by Jones & Boswell (1922), using mature bulbs of var. Yellow Globe Danvers planted in October or stored through the winter, while Jones (1923), using mature bulbs of var. Australian Brown planted 22 Dec., first found flower initiation on 22 Feb. It seems, therefore, that in most of the experiments of Thompson & Smith the growing plants were subjected to day-length and temperature treatments previous to and during flower initiation; whereas under more normal conditions the effects of photoperiod and temperature during growth would generally be operative *after* flower initiation had occurred. In one experiment Thompson & Smith planted sets in October which had previously been stored at 40° F. for a year; the effects of temperature on flowering were unchanged, and there was again no effect of day length. No record is presented of the date of inflorescence emergence in these experiments.

Earlier work on the effects of storage temperature on bolting behaviour had been recorded by Boswell (1923) and Jones (1927). Hungarian onion growers are said to store sets intended for their own use at a temperature gradually increasing from 20 to 35° C., the treatment extending over a period of at least 6 weeks before planting. This is said to result in a complete absence of bolting.

The effects of photoperiod upon *bulbing* and *ripening* have been extensively investigated, and are reviewed by Magruder & Allard (1937). Bulb development occurs in response to the stimulus of long days and the critical day length varies considerably (from about 12 to 16 hr.) for different varieties. Day lengths in excess of the minimum needed for bulbing hasten ripening. Apart from the results of Thompson & Smith (1938) the literature yields little information as to the effect of photoperiod upon *flowering* in the onion.

The main experiments in the present work were designed to investigate the effects upon flowering behaviour and also on bulbing and ripening of the following factors and their interactions:

(1) Time of germination of seed for sets (day-length and temperature effects).

(2) Temperature at which the sets are grown from seed.

(3) Temperature during different parts of the storage period.

(4) Temperature during the second season.

(5) Day length during the second season.

These should adequately survey the effects of temperature and day length, and provide a basis for detailed work. Attention was paid to the initiation

of inflorescence primordia and other changes in the internal morphology of the sets during the storage period and second season. This aspect of the work will be described in a separate communication.

In addition to the main experiments a preliminary experiment to study the Hungarian method of heat treatment was carried out using imported sets, in order to obtain some information in the first season. This experiment is briefly considered in the following section.

Experimental

I. *Preliminary experiment on heat treatment*, 1940

Commercial sets were obtained from four sources in Greece, France and Cyprus during Feb. 1940. From each lot, small and large size classes were selected. The 'small' classes ranged from $\frac{1}{2}$–$\frac{5}{8}$ in. diam. to $\frac{9}{16}$–$\frac{3}{4}$ in., and the 'large' from $\frac{5}{8}$–$\frac{13}{16}$ to $\frac{13}{16}$–1 in. for the different sources. Duplicate samples of forty were allotted to each of three storage treatments, viz. 5 weeks at a laboratory temperature of 10–20° C. (treatment *K*); 1 week at a temperature rising from 25 to 30° C. followed by 3 weeks at 30° C. and then 1 week at laboratory temperature (treatment T_m); the same, but with the fifth week at 35° C. instead of laboratory temperature (treatment T_h). The sets from all treatments were planted in the field on 12 Apr.

Both during and after the storage treatments, a small number of dissections was made periodically using spare sets from all four sources stored at laboratory temperature. The first microscopically visible signs of inflorescence initiation are an elongation of the central axis bearing the terminal growing point and the development of a spathe. These signs were first detected on 9 Apr. in sets from France, on 15 Apr. in sets of the two Greek lots, and on 7 May in a set from Cyprus. The highest incidence of bolting was in the French sets (see below).

The experiment yielded the following statistically significant results:

(1) The higher the storage temperature the greater was the loss of weight in store.

(2) Small sets had a mean loss in weight of 15·3 % as against 12·0 % for the large sets ($P < 0{\cdot}001$).

(3) There was a marked varietal effect on loss in weight, and the two Greek lots with 11·6 and 12·3 % mean losses respectively were significantly below both the French (15·0 %) and Cyprus (15·6 %) sets.

(4) Treatment T_m (25–30° C.) reduced bolting to a mean of 3·4 % as compared with 8·1 % following the laboratory-temperature storage treatment (*K*).

(5) Treatment T_h (25–35° C.) further reduced bolting to a mean of 1·4 %.

(6) Large sets gave a much higher incidence of bolting (mean 7·3 %) than small (mean 1·3 %).

(7) French sets bolted more (mean 7·9 %) than those from the other three sources (means 2·4–3·8 %).

There were no indications of any considerable interactions between the factors temperature, size and source.

Even in the laboratory-temperature treatment the incidence of bolting was not very high in this experiment, the highest value being 20 % for the large French sets. It is interesting to make a comparison between the small untreated Greek II sets (graded $\frac{9}{16}$–$\frac{3}{4}$ in. diam.), none of which flowered in the experiment, and a batch of 180 of the same sets graded $\frac{5}{8}$–$\frac{13}{16}$ in. diam. (i.e. very slightly larger) planted a month earlier in a garden. The latter showed 35 % bolting, indicating that time of planting might have a profound effect upon flower development, although since the comparison was not controlled for nutritional effects these might also have been involved. This suggestion of the importance of day-length and temperature effects has been confirmed by later work (Heath, 1943).

A number of malformed inflorescences appeared, all except one being from heat-treated sets. These malformations were of four kinds:

(1) The scape was stunted and bent over at the top, the inflorescence itself failing to emerge properly from the surrounding leaves. The incidence of this type was: two sets in treatment T_h (25–35° C.), one in treatment T_m (25–30° C.) and one in treatment *K* (laboratory temperature).

(2) The scape was normal and the spathe elongated into a green hooked leaf-like structure about 1 in. long, the inflorescence being small but otherwise normal. Two of these occurred, one in treatment T_h and the other in treatment T_m.

(3) The spathe was elongated into a structure resembling a foliage leaf 1 ft. or more in length, the inflorescence being small but otherwise normal and emerging late. One of these occurred in treatment T_h.

(4) The spathe was elongated as in (3) but the inflorescence was replaced by a single bulbil (Pl. 3, figs. 1, 3), which gave rise to another scape bearing an inflorescence inside the elongated spathe (Pl. 3, figs. 2, 4). Three examples of this type occurred, one in treatment T_h and two in treatment T_m.

The final yield data were of little value owing to the high incidence of fungal diseases.

II. *Main experiments*, 1940–1

(*a*) *Design of experiments and symbols used*

The experimental treatments are summarized in Table 1 together with the symbols by which they are denoted.

Five factors were investigated. For the first of these, time of germination, a series of five sowings (*I–V*) was made at intervals such that the germination dates should be separated by about $1\frac{1}{2}$ hr. of day length. The seedlings were grown in duplicate lots (A and B) at two temperature ranges—high temperature (*F*) in a greenhouse and low (*f*) in a

cold frame and later out of doors. The duplicates were kept distinct throughout the 2 years' experiment and therefore provided a valid basis for the estimate of 'error' due to uncontrolled causes operating during any part of the experimental period. Treatment *Vf* failed to produce sets. Sets from all other combinations of the first two factors were subjected to ten different storage treatments as in Table 1. In the second season, sets from all the ninety combinations of the first three factors were planted in the field. This field experiment thus consisted of a 9×10 factorial design with twofold replication, but by omitting the *VF* lots from the calculations more complete symmetry was achieved in a three-factor $4 \times 2 \times 10$ design. Sets from certain of the treatment combinations, namely, *I*, *III* and *IV*; *f* and *F*; T_3, T_4, t_3 and *C* in all possible combinations, were also planted out in a greenhouse at two levels of temperature (*H* and *h*) and two levels of day length (*D* and *d*). The greenhouse experiment thus consisted of an orthogonal five-factor $3 \times 2 \times 4 \times 2 \times 2$ design (i.e. 96 treatments) with two-fold replication. The results are presented in another paper (Heath, 1943).

in bulb forcing trays $12 \times 13\frac{5}{8} \times 5$ in. deep at the edge. The bottoms of the trays were raised along the centre line to improve drainage, the depth here being $4\frac{1}{4}$ in. Each box was filled so that the compressed soil was 1 in. below the top of the box, a gauge being used. The soil was well watered and the boxes left overnight before sowing. Seeds were placed singly on the soil spaced $0{\cdot}7 \times 0{\cdot}7$ in., giving 323 seeds per box, and were uniformly covered with $\frac{1}{4}$–$\frac{3}{8}$ in. of finely sifted soil pressed down and adjusted by the gauge. The boxes were lightly watered and covered with glass until the seedlings appeared.

TABLE 1. *Main experiments*, 1940–1. *Treatments and symbols*

Factor	Levels	Symbols
1. Time of germination	26. ii. 40 ($10\frac{1}{2}$ hr. day) for *F*	*I*
	8. iii. 40 ($11\frac{1}{4}$ hr. day) for *f*	
	18. iii. 40 (12 hr. day) for *F*	*II*
	18. iii. 40 (12 hr. day) for *f*	
	12. iv. 40 ($13\frac{1}{2}$ hr. day) for *F*	*III*
	10. iv. 40 ($13\frac{1}{2}$ hr. day) for *f*	
	6. v. 40 (15 hr. day) for *F*	*IV*
	2. v. 40 (15 hr. day) for *f*	
	12. vi. 40 ($16\frac{1}{2}$ hr. day) for *F*	*V*
2. Temp. in first season	High (mean about 21° C.)	*F*
	Low (mean about 14° C.)	*f*
3. Storage temp.	30° C. for 2 weeks. 'Common' (mean 12·2° C.) for 20 weeks	T_1
	30° C. for 8 weeks. 'Common' (mean 12·5° C.) for 14 weeks	T_2
	30° C. for 22 weeks	T_3
	'Common' (mean 12·1° C.) for 14 weeks. 30° C. for 8 weeks	T_4
	'Common' (mean 12·2° C.) for 20 weeks. 30° C. for 2 weeks	T_5
	'Common' (mean 12·1° C.) for 14 weeks. 25° C. rising to 35° C. over period of 8 weeks	T_6
	0–1·5° C. for 8 weeks. 'Common' (mean 12·5° C.) for 14 weeks	t_2
	0–1·5° C. for 22 weeks	t_3
	'Common' (mean 12·1° C.) for 14 weeks. 0–1·5° C. for 8 weeks	t_4
	'Common' (mean 12·5° C.) for 22 weeks	*C*
4. Temp. in second season	High (mean 26° C.)	*H*
	Low (mean for first 9 weeks 15° C.)	*h*
5. Day length in second season	Long (16 hr.)	*D*
	Short ($12\frac{1}{2}$ hr.)	*d*

Notes. Factors 4 and 5 were greenhouse treatments and were only applied to *I*, *III* and *IV*; for *F* and *f* combined with T_3, T_4, t_3 and *C*. Five-factor experiment (see Heath, 1943).

Vf failed to produce sets. All other combinations of factors 1, 2 and 3 planted out of doors under natural conditions of day length and temperature. Three-factor experiment.

(*b*) *Production of sets from seed*

Seed of var. Ailsa Craig (Prize Taker Stock) supplied by Messrs Ryder was sown in soil contained

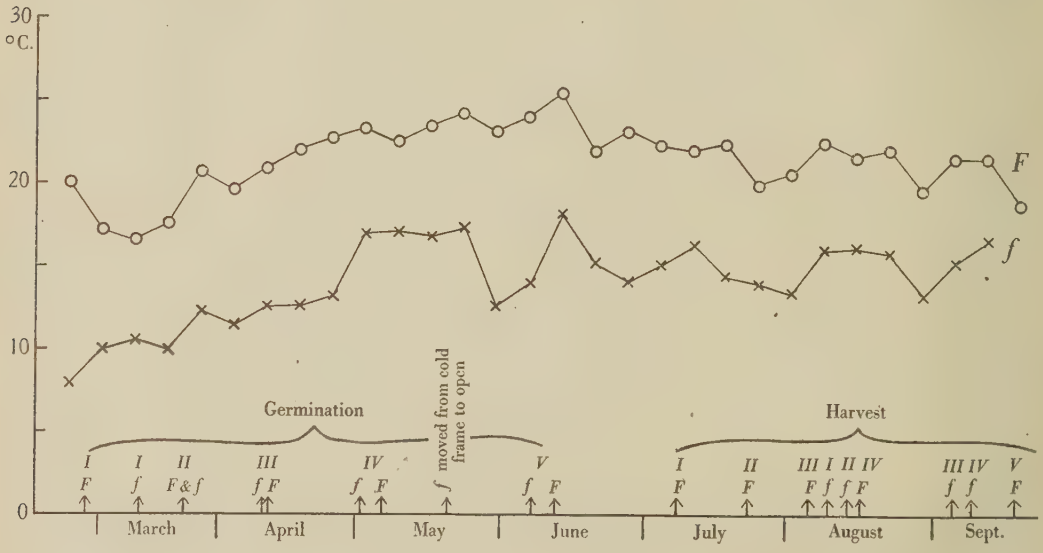
Half of the boxes sown were allotted at random to each of the duplicate lots A and B. Random arrangements were used both in the greenhouse (*F*) and frame (*f*) and the boxes were rearranged periodically to equalize positional effects.

Systematic germination counts were made on all boxes until such time as approximately 45% of the total number of seeds had germinated; this date was recorded as the 'date of germination'. Owing to the large temperature difference between *F* and *f* for the first sowing, the *IF* boxes gave a germination date 11 days earlier than *If*. For subsequent sowings the attempt was made to adjust the sowing dates so that the date of germination should be the same for *F* and *f*. The dates of germination are shown in Table 1, and are also indicated in Text-fig. 1 which

shows the weekly mean air temperatures for *F* and *f* obtained by the integration of thermograph records. *F* and *f* differed in respect of other conditions besides temperature, notably light intensity and atmospheric humidity, especially after the *f* boxes were moved out of the frame into the open on 20 May 1940, but it seems probable that the temperature difference constituted the most important factor. The *f* boxes, when in the open, were covered by Windolite screens during rain and the soil-moisture content was maintained similar to that of the *F* boxes by watering. Some seedlings were lost with damping-off due to *Rhizoctonia* sp. in *If*, *IIf* and *IIIf* during March and April, and losses due to *Fusarium* sp. occurred in *IF* and *IIF* towards the end of the season.

for *f*. Dissections on *IF* seedlings through the season indicated that until bulbing began there were generally three developing leaves at the growing point in addition to those which had emerged from the surrounding bases of the older leaves. With the onset of bulbing the emergence of further leaves practically ceased in *IF* plants, but the number of unemerged leaf or scale primordia increased to a maximum of about ten.

The onset of bulbing was recorded as the date when all the boxes of a treatment first showed a number of plants definitely swelling at the base. These data revealed that in the three first sowings *I–III*, both in *f* and *F*, bulbing (as estimated) began on approximately the same date, viz. 25 May, when



Text-fig. 1. Main experiments 1940. Growth of sets from seed. Weekly mean air temperatures from thermograph records.

It was desired to ensure a supply of sets of known leaf number for purposes of dissection, and as death of leaves normally makes counts impossible at harvest, every alternate leaf was marked during the season on a large number of seedlings with indian ink or sealing-wax varnish. At first the attempt was made to mark leaves on *all* plants in four boxes out of each lot (e.g. *IVf*A), but as the seedlings became larger it was found impossible to mark those in the middle of the box without damaging the others. Finally, therefore, only plants near the edge of the box could be marked. Although regular counts were not made through the season, the dates were noted when *all* the boxes in any one treatment (e.g. *IF*) first showed several plants with the next leaf number.

These data indicated that until the onset of bulbing the interval between the emergence of successive leaves was about 12 days for *F* and about 16 days

the day length (sunrise to sunset) was 16 hr. In *IV*, bulbing was delayed, but again occurred on approximately the same date both in *F* and *f* (30 May). In *V* bulbing occurred about 1 July (16 hr. 33 min.) in both *F* and *f*, the plants having been in long days, more than 16⅛ hr., since germination. Some plants were noted as bulbing in which only the first foliage leaf had emerged from the sheathing base of the cotyledon. These results, together with the number of days from germination to bulbing and the approximate number of emerged foliage leaves, are shown in Table 2. Under the prevailing conditions of increasing day length, for *I–III* bulbing onset *as estimated* was completely determined by day length and was unaffected by temperature differences such as those between *F* and *f* (cf. Text-fig. 1). The data for *IV* and *V* indicate that a minimum leaf number of 1 or 2 was needed before bulbing could begin.

Although temperature apparently had no effect on bulbing onset, the effect on ripening was marked. In *F* most of the plants showed the normal symptoms of incipient dormancy, the tissues of the neck softening and the leaves collapsing and finally dying, without any deliberate reduction of the soil moisture. When the leaves of nearly all the plants for any sowing (e.g. *IIF*) had fallen over, all such plants were harvested, and the dates are shown in Table 2. The rest of the plants were harvested about a week later, and the number of plants which had to be discarded as thick-necked or not bulbed was negligible except in *VF*. In *VF*A, 20·5 % and in *VF*B 18·2 % of the plants were discarded for these reasons. The sets in *f* showed no tendency to ripen naturally, and as they were considerably larger than the sets in *F*, owing probably to the longer growth period coupled with the higher light intensity, an attempt was made to ripen them by allowing the soil to dry out. Watering was therefore stopped from 29 July for *If* and *IIf*, 19 Aug. for *IIIf*, 24 Aug. for *IVf* and 7 Sept. for *Vf*. The sets were harvested when most of the leaves had died, generally about a fortnight after watering ceased, but in many cases the dead leaves remained more or less erect. The numbers of *f* plants which had to be discarded as not bulbed or as seriously thick-necked were unfortunately not noted for *If* and *IIf*. For *IIIf* they were 19·8 %, for *IVf* 45·7 %, while in *Vf* only 8·6 % of the A plants and 5·6 % of the B plants made bulbs (as compared with 79·5 and 81·8 % in *VF*): *Vf* was therefore discarded. The sets from *F* and from *f* were cured in trays, in the sun when possible, and the tops twisted off. They differed greatly in general appearance, the *F* sets being smaller, better coloured, thinner skinned and almost entirely free from thick-necks.

When the sets in each treatment (e.g. *IIIf*) were cured, all the unmarked sets of either the A or B lot were graded in weight classes of 1 g., the 0–1 g. class being further subdivided as seemed necessary. Some of the extreme classes were then discarded, being so chosen that each of the rejected 'tail' portions of the distribution amounted to *about* 5 % of the total. All unmarked sets falling outside these chosen weight limits were also discarded from the other duplicate, and the same limits were applied to the leaf-marked sets of both A and B. In this way the more extreme variability in size was reduced while the other main characteristics of the samples were retained.

The weights and leaf numbers after discarding the extremes are shown in Table 3. The leaf-marked sets were in all cases heavier than the corresponding unmarked sets, especially before removal of the extremes. This was probably due to the marked sets having occupied the edges of the boxes, but there may also have been a tendency to overlook smaller plants when marking leaves. It is clear that the marked-leaf sets did not constitute truly representative samples of the lots from which they were taken, though among themselves they were comparable. The leaf numbers, which were scarcely affected by removal of the extremes, show for *F* a progressive fall with sowing date, and a comparison with Table 2 suggests that in *F* leaf emergence practically ceased with the onset of bulbing (except possibly in *IVF* and *VF*). In *f*, on the other hand, the final mean leaf numbers as shown in Table 3 are almost constant and here considerable leaf emergence occurred after bulbing onset. The mean weights given in Table 3 show that the *f* sets were in all cases much heavier than the corresponding *F* sets. The agreement between duplicates is in most cases good throughout the table.

TABLE 2. *Main experiments*, 1940–1. *Onset of bulbing in seedlings*, 1940

Approximate dates when all boxes first showed some plants definitely bulbing. Also harvest dates

(For symbols see Table 1, p. 211)

	I		*II*		*III*		*IV*		*V*	
	F	*f*	*F*	*f*	*F*	*f*	*F*	*f*	*F*	*f*
Bulbing:										
Date	25. v	25. v	25. v	25. v	25. v	25. v	30. v	30. v	1. vii	1. vii
Day length	16 hr.	16 hr.	16 hr.	16 hr.	16 hr.	16 hr.	16 hr. 14 min.	16 hr. 14 min.	16 hr. 33 min.	16 hr. 33 min.
Days from germination	88	78	68	68	43	45	24	28	19	24
No. of leaves	6	4	5	3–4	4	2–3	2	2	1–2	1–2
Harvest:										
Date	8. vii	9. viii	23. vii	13. viii	5. viii	5. ix	15. viii	9. ix	18. ix*	—
Days from germination	132	154	127	148	115	148	101	130	98	—

* Actual date 25 Sept., but most of sets had been dead ripe for a week.

(*c*) *Storage treatments*

The weights given in Table 3 were obtained as soon as possible after curing and grading was complete and therefore at widely differing times, ranging from the beginning of August for *IF* to the beginning of October for *VF* and *IVf*. The unmarked sets were allotted to the ten storage treatments (see Table 1)

and the separate lots (e.g. *IIf* T_3A) were weighed on 4–10 Oct. 1940 at the beginning of storage. The leaf-marked sets were also weighed on 4–10 Oct. 1940 and a random sample of thirty covering all leaf numbers was taken from each duplicate lot of each time-temperature combination (e.g. *IIIF*B). These samples were pickled in 50% alcohol on 10–12 Oct. 1940 at the beginning of the storage period to provide material for dissections. The remainder of the leaf-marked sets was allotted to the ten storage treatments in almost equal numbers in approximately the proportions in which they occurred; e.g. if in *IIf*B there were available twenty 4-leaf, eighty-two 5-leaf and sixty 6-leaf sets, each storage treatment would receive 2, 8 (or 9) and 6 respectively. Subject to these restrictions they were allotted in a random manner. These marked-leaf sets were subjected to the same storage conditions as the unmarked sets. At the end of the full treatment period of 22 weeks half the leaf-marked sets from each lot were pickled for dissection on 13–15 Mar. 1941. The remainder were kept under the 'common' storage (*C*) conditions until shortly after the planting of the greenhouse treatment sets and were then also pickled (29 Apr.–1 May 1941).

The high-temperature treatments T_1 to T_5 consisted of storage for the stated periods (Table 1) in net bags on wire-netting trays in a large incubator at 29–32° C. A small current of air was drawn by a filter pump over the heating elements and then through the incubator. The bags were arranged on the trays in a random order and the positions of the trays in the incubator were re-randomized weekly. The T_6 treatment was intended to simulate the Hungarian method of heat treatment (see p. 209), and the sets were stored for the last 8 weeks of the storage period in perforated zinc trays in an incubator with warm air blowing through. The positions were re-randomized weekly, and the temperature was raised gradually from 24–28° C. for the first week to 33–36° C. for the eighth week. Owing to a breakdown in the fan supplying the warm-air current the heat was off for 3 days during the second week.

The low-temperature treatments t_2 to t_4 consisted of storage for the periods shown in Table 1 in a large refrigerator in which the air was circulated by a fan. The sets were in net bats on wire-mesh trays, and the temperatures in different parts of the refrigerator and at different times varied between 0 and 1·5° C. The positions of the trays were re-randomized weekly.

The 'common' storage treatment *C* consisted of storage on perforated zinc trays in racks in a double-walled wooden shed, with slight heating in very cold weather. During the 22 weeks covered by the various treatments the weekly mean temperatures in this shed varied between 9·5 and 16·8° C., the means for the various periods of 'common' storage being 12·1–12·5° C. (see Table 1). After the first 6 weeks of the storage treatments (i.e. on 22 Nov. 1940) the shed was completely darkened, so that from this date the conditions were comparable with *T* and *t* in this respect. At the end of the storage treatments (12 Mar. 1941) all sets were kept under the 'common' storage conditions until planting (7–9 Apr. 1941 for the field planting and 16–17 Apr. 1941 for the greenhouse treatments). During this period the weekly mean temperatures varied from 8·3 to 11·9° C.

The various lots of unmarked sets were re-weighed when their storage conditions were changed (e.g. *III*f_2A after 8 weeks). *All* the lots were weighed after 14 weeks and again after 22 weeks at the end of the storage-treatment period. The control lots were also weighed after 2, 8 and 20 weeks.

A few sets noted as decayed during storage were discarded before the final weighings, and were allowed for in calculating mean weights. Shrivelled sets were not discarded during the storage period and, therefore, loss in weight data included some sets which had dried out completely. Such sets were practically confined to the *FT* lots and especially

TABLE 3. *Main experiments, 1940–1. Weights (in g.) and leaf numbers of sets after discarding extreme classes*

(For symbols see Table 1, p. 211)

		F					*f*			
		I	*II*	*III*	*IV*	*V*	*I*	*II*	*III*	*IV*
Unmarked sets:										
Mean weight	A	2·45	1·67	1·43	1·29	1·14	3·81	3·33	3·18	2·61
	B	2·33	1·74	1·41	1·25	1·22	3·82	3·59	3·15	3·04
Range	A	0·7–6·0	0·5–4·0	0·35–4·0	0·35–3·5	0·35–3·5	1·0–8·0	1·0–7·0	1·0–7·0	0·7–7·0
	B	0·7–6·0	0·5–4·0	0·35–4·0	0·35–3·5	0·35–3·5	1·0–8·0	1·0–7·0	1·0–7·0	0·7–7·0
Leaf-marked sets:										
Mean weight	A	2·76	2·14	1·80	1·47	1·63	5·06	4·03	3·80	2·75
	B	2·81	1·76	1·84	1·60	1·64	5·02	4·23	4·04	3·59
Mean leaf no.	A	6·02	5·49	4·08	3·26	3·08	5·56	5·58	5·64	5·19
	B	5·92	5·29	4·28	3·52	3·15	5·54	5·69	5·91	5·27
Range for leaf no.	A	5–8	4–8	3–7	2–6	2–6	4–8	4–8	4–8	4–7
	B	5–8	4–7	3–7	2–5	2–6	4–8	4–8	3–8	4–8

occurred in FT_3. This is reflected in the very high losses in weight of such lots (see Table 4).

The data for percentage loss in weight over the 22 weeks' storage period were examined by the method of analysis of variance. For this purpose VF was omitted in order to give symmetrical tables, and the six T treatments were analysed separately from the other four treatments. This separation into two groups of treatments was necessary because the variances 'within treatments' differed considerably between the two groups although they did not differ greatly within the groups. The procedure was first to test the three-factor interaction against the random variance derived from the duplicates, and if found significant to use this three-factor interaction as 'error' variance for testing the two-factor interactions. These in turn, if significant, were used as 'error' for testing the corresponding main effects.

Table 4 shows the analysis of variance for loss in weight in 22 weeks' storage in the T treatments. The *average* effect of time of germination (*I–IV*) is not significantly greater than the three-factor interaction in which it is involved, although testing against the random variance shows that in this particular set of experimental treatments there was a real average effect ($P<0{\cdot}05$). The temperature during the first season (*Ff*), on the other hand, has a very significant main effect, overshadowing any differences in its effect at different levels of the other factors. It is, therefore, of general interest. The mean percentage losses over all levels of the other factors were *F*, 41·8%; *f*, 25·0%, i.e. the smaller and thinner-skinned *F* sets had a much greater loss in weight under heat treatments than the *f* sets. The average effect of storage treatment T_1–T_6 is also very significant when compared with its interactions, and the treatment means over all levels of the other factors are, therefore, of interest. These are: T_1, 21·2%; T_2, 35·1%; T_3, 55·7%; T_4, 34·3%; T_5, 22·1%; T_6, 32·1%. The difference required between two such means to give a significance of P 0·05 is 13·3. T_1 and T_5 do not differ significantly nor do T_2 and T_4. The results are therefore consistent with the hypothesis that the effects of the 30° C. and 'common' storage temperatures are purely additive. T_6 is not significantly different from T_4, but all differences between pairs of treatments involving longer and shorter exposure to high temperature are either significant or approach the P 0·05 level.

Of the two-factor interactions, only that between temperature in the first season *Ff* and storage treatment T_1–T_6 is significant when tested against the three-factor interaction. In view of this significant two-factor interaction it is necessary to examine the treatment (T) effects within *F* and *f* separately, and such examination reveals that the above conclusions as to storage-temperature effects still apply. The *F* minus *f* difference is also large and significant within each T treatment. The interaction apparently consists in the much greater increase in percentage loss with more prolonged heat treatment in *F* sets than in *f* sets (see Table 4).

Since the variance due to the three-factor interaction is significantly greater than the random variance, the treatment effects within each time-temperature combination must also be considered. In *f*, the T_1 minus T_5 and T_2 minus T_4 differences are not significant for any of the four germination times. In *F*, however, the effects are much less regular, and whereas in *IIF* the loss in T_4 (49·8%)

TABLE 4. *Main experiments*, 1940–1. *Percentage loss in weight during 22 weeks' storage treatment. High-temperature treatments* (T)

(For symbols see Table 1, p. 211)

Analysis of variance

Variation due to	Degrees of freedom	Variance	Variance ratio	With	*P*
Time of germination *I–IV*	3	78·4	1·53	*I–IV* v. *Ff* v. T_1–T_6	>0·20
Temperature *Ff*	1	6695·0	31·18	*Ff* v. T_1–T_6	<0·01
Storage treatment T_1–T_6	5	2504·1	11·66	*Ff* v. T_1–T_6	<0·01
Interaction: *I–IV* v. *Ff*	3	32·3	—	*I–IV* v. *Ff* v. T_1–T_6	—
Ff v. T_1–T_6	5	214·7	4·21	*I–IV* v. *Ff* v. T_1–T_6	<0·05
I–IV v. T_1–T_6	15	46·9	—	*I–IV* v. *Ff* v. T_1–T_6	—
I–IV v. *Ff* v. T_1–T_6	15	51·0	2·03	Random	<0·05
Random	48	25·1	—	—	—

Mean percentage loss over all germination times (*I–IV*)

	T_1	T_2	T_3	T_4	T_5	T_6
Temperature in first season: *F*	25·5	46·3	69·4	42·9	26·8	39·6
f	16·8	23·8	42·0	25·7	17·3	24·5

S.E. of a single mean = 2·5
Significant difference required for P 0·05 = 7·6
Significant difference required for P 0·01 = 10·5

is significantly greater ($P<0.01$) than that in T_2 (31·8%), in *IVF* the converse situation holds, T_2 (60·5%) showing a significantly greater loss than T_4 (41·2%). These two effects, which are difficult to account for, provide a large part of the three-factor interaction observed. The only other significant difference between two heat treatments of the same duration is the difference $IIFT_4$–$IIFT_6$ ($P<0.05$). The maximum loss (75·0%) is shown by the $IVFT_3$ treatment.

The data for the high-temperature treatments T_1–T_5 are therefore consistent with an additive hypothesis whether we consider means over all levels of the other two factors, means within *F* and *f* over all times *I–IV*, or within the individual times for *f* only. The data from *IIF* and *IVF* considered separately are opposed to such an hypothesis.

more rapid loss under 'common' storage conditions after cold storage than before it. This would account for the positive t_2 minus t_4 difference found. The effect, although highly significant statistically, is not large, the small random variance in this group of treatments making its detection possible.

The interaction between temperature *Ff* and time *I–IV* is such that whereas in *f* the loss in weight increases progressively from *I* to *IV* indicating the existence of a size effect (see Table 3), the *F* data show no such progressive change. In the former *IVf* is significantly above *If*, but in the latter *IF* is significantly above *IIIF* with *IIF* and *IVF* intermediate. There is thus no suggestion that size has had an important effect on the differences in loss of weight among the *F* sets. The data are given in Table 5.

TABLE 5. *Main experiments*, 1940–1. *Percentage loss in weight during 22 weeks' storage treatment: Cold storage (t) and 'common' storage (C)*

(For symbols see Table 1, p. 211)

Analysis of variance

Variation due to	Degrees of freedom	Variance	Variance ratio	With	*P*
Time of germination *I–IV*	3	1·86	—	*I–IV* v. *Ff*	—
Temperature *Ff*	1	124·05	33·7	*I–IV* v. *Ff*	0·01
Storage treatment *C–t*	3	151·24	141·4	Random	<0·001
Interaction: *I–IV* v. *Ff*	3	3·69	3·45	Random	<0·05
Ff v. *C–t*	3	2·42	2·26	Random	>0·05
I–IV v. *C–t*	9	1·19	1·11	Random	>0·20
I–IV v. *Ff* v. *C–t*	9	1·26	1·18	Random	>0·20
Random	32	1·07	—	—	—

Mean percentage loss over all storage treatments (t_2–t_4, C)

	Time of germination			
	I	*II*	*III*	*IV*
Temperature in first season: *F*	14·1	13·5	12·6	13·4
f	10·0	10·4	10·6	11·4

S.E. of a single mean = 0·37
Significant difference required for *P* 0·05 = 1·1
Significant difference required for *P* 0·01 = 1·4

A similar analysis carried out on the data from t_2, t_3, t_4 and *C* is simpler, since only one interaction, that between temperature *Ff* and time *I–IV*, is significant (see Table 5). There is no evidence that the average effect of time of germination (*I–IV*) is real, whether it is tested against this interaction or the random variance, but the *Ff* main effect is very significant, the means being *F*, 13·4%; *f*, 10·6%. The average effect of storage treatment *C–t* is highly significant, the treatment means being: *C*, 15·0%; t_2, 13·5%; t_3, 7·9%; t_4, 11·5%. The required difference between two such means for *P* 0·01 is 1·0, and, therefore, all the differences are real. The greater loss in t_2 than t_4 thus apparently disproves that an additive law applies to the 'common' and cold-storage conditions. The type of departure from an additive hypothesis suggested by these data is a

The available data for loss in weight during the different parts of the storage period were examined to elucidate the apparent departure from an additive law in the case of t_2 and t_4. The loss in weight in the *C* treatment for the first 14 weeks, corresponding to the 'common' storage period for t_4, was 7·8%, while that for the last 14 weeks, corresponding to the 'common' storage period for t_2, was 10·6%. The difference between these two values, namely, 2·8, agrees reasonably well with the difference of 2·0 found between t_4 and t_2. If account is also taken of a small and statistically non-significant increase in rate of loss for the later part of the storage at 0° C. of the t_3 sets, the agreement is considerably improved. Thus, the apparent departure from an additive hypothesis in the case of t_2 and t_4 may be explained satisfactorily by the greater loss under

'common' storage conditions in the later part of the storage period, and there is no reason to suppose that an 'after effect' of the low-temperature treatment affected loss under the 'common' storage conditions.

(*d*) *Behaviour in the field in second season. Three-factor experiment*

At the end of March 1941, thirty sets were taken at random from each separate lot, e.g. *IIIF*T_4A, for field planting. Owing to losses in the T_3 lots from decay or complete drying up of sets, however, it was necessary to take from most of the FT_3 and two of the fT_3 lots only twenty to twenty-six sets, or in one case only thirteen, in order to leave twenty good sets for the greenhouse planting. These numbers included some much more shrivelled sets than were retained for the greenhouse treatments, since a full stand for the latter was most important. The T_3 treatments in the field were thus somewhat penalized as far as percentage survival was concerned (see below). The sets were planted 7–9 Apr. 1941, in heavy, unmanured soil which had been newly dug up from grass the previous autumn, spaced 4 × 15 in. The 180 plots of thirty sets were distributed over the field in a random arrangement.

The plants came up satisfactorily, but no 'germination' counts were made owing to the wet condition of the soil at the time. A prolonged cold spell followed which, combined with the unfavourable soil conditions, made early growth very slow. A number of plants succumbed, especially among the FT_3 lots, as was revealed by counts made on 4–5 June 1941, when the first few inflorescences had emerged on some of the *f* plots.

The data for percentage of plants surviving to 4–5 June 1941 are like all the data considered in the present section, except the weights at harvest, in that the individual plot figures are percentages based on small numbers. Such data are not normally distributed and must be transformed in order that the 'within treatment' variances may be approximately independent of the treatment means. The appropriate transformation consists in substituting for the original percentages the angles whose sines are the square roots of the ratios. The analysis of variance is then carried out on these angular values (see Cochran, 1938).

Analysis of the percentage survival data for 4–5 June 1941 (after angular transformation) reveals a very large and highly significant average effect of growth temperature *Ff* ($P<0{\cdot}001$), the arithmetic means of the untransformed percentages being *f* 84·3%, *F* 66·8%. Thus, the *f* sets on the average survived the adverse conditions considerably better than the *F* sets. Average effects of time of germination and of storage temperature are highly significant ($P<0{\cdot}001$) when tested against the random variance, but the first-order interactions with growth temperature (*Ff*) are also very significant. The type of hypothesis disproved by a significant interaction in terms of such transformed data is without physiological meaning, but the original percentages reveal that in the case of storage temperature the interaction is also large and consistent in terms of the untransformed values, and it is therefore of more interest to examine the storage-temperature effects within *F* and *f* separately. The treatment means of the untransformed percentages are shown in Table 6. It is not possible to give a standard error in terms of such untransformed percentages, but significant differences between storage treatments are indicated at the foot of the table. These show that whereas in *f* there is evidence only that the cold-storage treatments t_3 and t_4 differ from any others and none that the heat-treated sets are worse than those from 'common' storage (*C*), in *F* not only are the T_3 sets very much worse than any others* but the T_6 sets also give significantly worse results than *C*. The *F* data indicate a generally harmful effect of heat treatment, increasing with longer exposure to high temperature, while the *f* data give no such indications. In both *F* and *f* the cold-storage treatments t_3 and especially t_4 appear to have beneficial effects, although the difference from *C* does not quite reach the *P* 0·05 level of significance for ft_3 or Ft_4 and is well short of it for Ft_3. The t_2 treatment, on the other hand, appears to have no effect in enhancing survival. The *f* minus *F* difference for corresponding storage treatments reaches significance for $T_2T_3T_4T_6$ and t_2 but

TABLE 6. *Main experiments*, 1941. *Three-factor experiment. Percentage survival in the field on 4–5 June 1941. Two months after planting*

(For symbols see Table 1, p. 211)

Means over all times of germination

	T_1	T_2	T_3	T_4	T_5	T_6	C	t_2	t_3	t_4
f	78·3	80·8	86·5	77·5	84·2	77·9	82·9	88·8	92·5	93·8
F	73·7	58·8	23·5	61·3	70·8	56·7	77·1	72·5	84·6	88·8

Significant differences ($P<0{\cdot}05$):

In *f*: t_4 > all except t_3 t_2 and T_3.
t_3 > T_1 T_2 T_4 and T_6.

In *F*: t_4 and t_3 > all except *C*.
T_3 < all others.
T_6 < T_1 and *C*.

* Part of this effect was due to the non-random nature of the samples of T_3 sets planted.

not for the other treatments, although the F value is in all cases the lower. The mean percentages for the germination-date series, taken over all levels of the other factors, are: *I*, 85·0; *II*, 77·7; *III*, 72·2; *IV*, 67·4. Of these *I* is significantly above *II* which is significantly greater than *III* and *IV*. This effect is probably due to size differences.

After the first count on 4–5 June 1941, further counts of the numbers of plants per plot with and without inflorescences were made on 16 and 30 June and 28 July 1941. The count on 30 June 1941 was made immediately after severe damage by rooks had occurred, many plants being broken, pecked up or even completely removed. Up to this date occasional losses had occurred both from birds and from wireworm, but this last was so severe as greatly to increase the random variation in the experiment. In view of this damage, where the counts for 30 June the adjusted data are discussed below. The T_2, T_3 and T_4 treatments were omitted from the analysis for 16 June and the T_3 treatment from that for 30 June 1941 as flowering was either absent or negligible. For each of the three counts the average effect of storage treatment was very significant ($P<0{\cdot}01$). The treatment means of the untransformed percentages for the three occasions are shown in Table 7, and significant differences between treatments are again indicated at the foot of the table. The more prolonged heat treatments had marked effects in delaying and reducing flowering; the T_3 treatment prevented bolting entirely until a very late stage, and even then the incidence was only 2%. It is worthy of note that in the final count T_4 was significantly above T_2, thus disproving an additive hypothesis as to the effects of 30° C. and 'common' storage temperatures on total flowering. This is

TABLE 7. *Main experiments*, 1941. *Three-factor experiment. Percentage flowering in the field*

(For symbols see Table 1, p. 211)

Means over all times of germination (f only)

Date	T_1	T_2	T_3	T_4	T_5	T_6	C	t_2	t_3	t_4
16 June	8·8	0·5	0·0	0·7	9·7	2·1	10·4	5·1	3·3	13·2
30 June*	9·5	3·7	0·0	4·5	14·8	5·1	13·6	5·6	6·6	21·8
28 July*	11·1	3·9	2·2	11·3	15·6	5·1	13·9	5·9	6·7	22·1

Significant differences ($P<0{\cdot}05$):

16 June: C and $T_5 > t_3$ and T_6 (also T_2, T_3 and T_4†).
$t_4 > t_2$, t_3 and T_6 (also T_2, T_3 and T_4†).
$T_1 > T_6$ (also T_2, T_3 and T_4†).

30 June: C and $T_5 > t_2$, t_3, T_2, T_4 and T_6 (also T_3†).
t_4 $> t_2$, t_3, T_1, T_2, T_4 and T_6 (also T_3†).

29 July: C and $T_5 > t_2$, t_3, T_2, T_3 and T_6.
t_4 $> t_2$, t_3, T_1, T_2, T_3, T_4 and T_6.
T_4 $> T_2$ and T_3.

* Where a plot shows a lower percentage than on a previous occasion owing to bird damage, the previous value has been used.

† Not included in the analysis.

or 28 July 1941 showed a decrease in the percentage flowering for any plot, the previously obtained percentage was inserted. Out of eighty f plot values, nine were so adjusted for 30 June and twenty-one for 28 July. This method causes some bias in the direction of increasing percentage flowering, but since the percentage was in most cases low this effect is unlikely to be large.

The most striking fact that emerged from the inflorescence counts was that the F sets were almost entirely free from bolting, only six of them flowering in the whole experiment and these being distributed among four different storage treatments. Statistical analysis for percentage flowering was therefore confined to the f data, for which the 'angular transformation', was used. Analysis of the data for 30 June and 28 July 1941 both before and after the adjustments mentioned, showed that the main conclusions were unaffected but that the reduction in error variance due to the adjustments in general increased the significance of treatment effects. Only unlike the case of the data for loss in weight during storage in which there was no evidence that the effects were other than additive. These results, therefore, give no indication that the reduction of total flowering by high-temperature storage is brought about by the drying effect, although the possibility is not precluded. Although the first visible signs of inflorescence initiation only occur at about the end of the storage period, early heat treatment (T_2) was more efficacious than late (T_4) in reducing bolting. The differences between T_1 and T_5 were also in the same direction, and for the last two counts approached but failed to reach the conventional P 0·05 level of significance. In the final count T_4 was not significantly below C, but the highest storage temperature, reached in T_6, caused a significant reduction in total flowering. Of the cold-storage treatments, both t_2 and t_3 were effective in reducing the incidence of flowering below the C treatment. Again, the effects of storage temperatures (0° C. and 'common') were not additive, for t_4 was

in all counts significantly above t_2 ($P<0{\cdot}01$). The actual values obtained for t_4 were in all counts also above those for C, though not significantly so. Collateral evidence from dissections suggests that this may be a real effect of late cold storage. The average effect of time of germination only reached significance in the analysis for the data of 30 June 1941, where the means over all storage treatments were *I*, 12·1%; *II*, 8·5%; *III*, 8·4%; *IV*, 5·1%, the *I* minus *IV* difference being significant ($P<0{\cdot}01$). This effect was probably due to the difference in size (cf. Table 3).

Owing probably to a hot dry spell from mid-June onwards, large numbers of bulbs ripened off prematurely during July, forming onions of extremely small size (about 6 g. in weight). On 28 July all such bulbs in which the leaves had completely died down is thus in the opposite direction to that in respect of bolting, but again an additive hypothesis is disproved and there is no indication that the results are brought about by the drying effect of heat treatment. There is no evidence that the cold-storage treatments affected the percentage of prematurely ripened bulbs.

The final harvest was carried out on 28 Aug., all remaining bulbs being lifted whether ripe or not. The tops were twisted off and the bulbs weighed without curing. Most of the bulbs were very small, with the exception of many of the T_3 bulbs which were of fair size though inclined to be thick-necked. (On one plot, fT_3IVA, the bulbs averaged 107 g. each.) The mean weights per bulb for the combined harvests of 28 July and 28 Aug. were subjected to analysis of variance. Only the average effects of first season temperature (*Ff*) and of storage treatment

TABLE 8. *Main experiments*, 1941. *Three-factor experiment. Percentage premature ripening in the field.* (*Counts of dead ripe bulbs on 28 July 1941*)

(For symbols see Table 1, p. 211)

Storage treatment means over all levels of other factors

T_1	T_2	T_3	T_4	T_5	T_6	C	t_2	t_3	t_4
68·7	71·2	6·1	30·6	61·0	40·0	73·0	69·5	64·9	69·2

Significant differences ($P<0{\cdot}05$):
T_4 < all except T_6 (and T_3*).
T_6 < all except T_4, t_3, t_4 (and T_3*).

* Not included in the analysis.

TABLE 9. *Main experiments*, 1941. *Three-factor experiment. Mean weights per bulb.* (*Combined harvests of 28 July and 28 Aug.* 1941)

(For symbols see Table 1, p. 211)

Storage treatment means over all levels of other factors

T_1	T_2	T_3	T_4	T_5	T_6	C	t_2	t_3	t_4
8·0	10·4	23·4	13·9	10·0	12·4	6·1	8·6	10·4	9·9

S.E. of a single mean = 2·51
Significant difference required for P 0·05 = 7·1
Significant difference required for P 0·01 = 9·4

were lifted, counted and weighed. It was clear that storage treatment had considerably affected their relative numbers. The percentages of prematurely ripened bulbs, after 'angular transformation', were therefore subjected to analysis of variance. The T_3 treatment was omitted from the analysis owing to the very much lower incidence of premature ripening than in any other treatment. The only significant effect was that for storage treatment ($P<0{\cdot}01$), and the treatment means of the untransformed percentages are shown in Table 8; again, significant differences between treatments are given. The T_3 treatment almost entirely prevented premature ripening, and the two prolonged and late heat treatments (T_4 and T_6) also had considerable effect in delaying ripening. The T_2 treatment, on the other hand, apparently had no such effect. The difference between T_2 and T_4 in respect of premature ripening were significant, P being about 0·001 in each case. The mean weight for the *f* onions was 14·0 g. and that for the *F* onions 8·7 g., these low values being largely due to the preponderance of premature ripening indicated in Table 8. The smaller bulbs from the *F* sets may have been due to their smaller initial weight. The mean weights for the storage treatments are shown in Table 9. The T_3 mean is almost four times that for 'common' storage (C) and is significantly above all others ($P<0{\cdot}01$), and the T_4 minus C difference is also significant ($P<0{\cdot}05$). The T_6 minus C difference approaches but fails to reach the P 0·05 level. These differences in mean weight are to be associated not only with the lower incidence of flowering, but even more with the delayed ripening of the T_3, T_4 and T_6 treatments (see Table 8), which in the rather abnormal season under discussion proved a distinct advantage. Owing to the great

irregularity produced by the bird and wireworm attacks, the data for yields on an area basis are unsatisfactory, but the two storage treatments giving the largest and smallest bulbs respectively, viz. T_3 and C, also gave the highest and lowest yield per unit area.

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EXPLANATION OF PLATE 3

Fig. 1. Onion plant grown from a heat-treated French set (treatment T_m), showing a malformation of the inflorescence; the spathe elongated into a foliage leaf-like structure and a swelling where a bulbil has developed in place of the flower head. June 1940.

Fig. 2. The same after the leaf-like spathe split, allowing the emergence of a small secondary inflorescence and a single abortive leaf, both produced from the bulbil. July 1940.

Fig. 3. Another plant as in Fig. 1. June 1940.

Fig. 4. The same after the leaf-like spathe has been opened, showing the small secondary inflorescence and three small leaves, all produced from the bulbil. July 1940.

(*Received* 19 *December* 1942)

Fig. 1

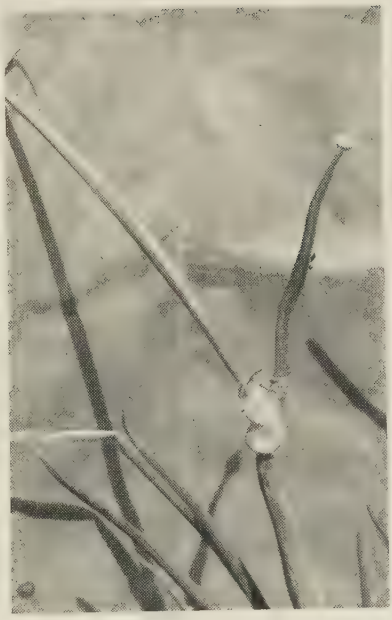

Fig. 2

Fig. 3

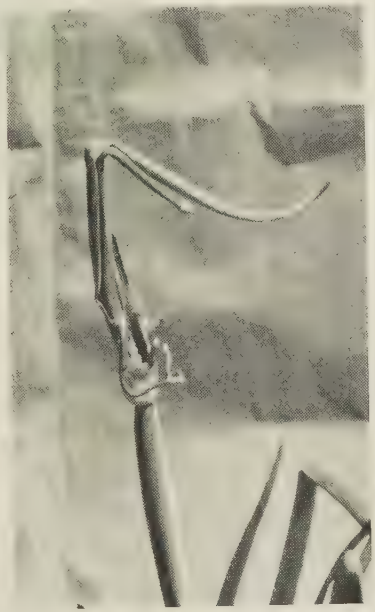

Fig. 4

Black scurf and stem canker of potato (*Corticium Solani* Bourd. & Galz.)

Field studies on the use of clean and contaminated seed potatoes and on the contamination of crop tubers

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Black scurf and stem canker of the potato was investigated in field trials on infected soil at Warburton, Cheshire, to study the effect of planting clean and contaminated seed, and the relation between the dates of planting and lifting and the contamination of the crop tubers.

Black scurf was prevalent on crops grown from clean seed but was more severe on crops raised from contaminated seed. Under conditions favourable for the disease the yields from clean and contaminated seed were satisfactory and were not significantly different. Young shoots of plants from contaminated seed were severely attacked and tuber formation was checked; these results were not obtained on clean seed plots.

More black scurf occurred on late-dug crops; on early-dug plots the disease, although almost absent in 1941, was prevalent in 1942. Late planting failed to reduce appreciably the amount of black scurf.

Variety trials included Arran Banner, Kerr's Pink (two vigorous varieties), King Edward and Majestic (two less vigorous varieties). All were heavily infected and each recovered well from an attack on the young shoots.

Inoculation of the seed at planting time did not affect the results; in all trials, misses and wilted shoots caused by *Corticium* were rare and there was no relation between the yield and the amount of black scurf on the crop tubers.

The results indicate that the disease causes little, if any, loss under farm conditions.

In this country black scurf and stem canker of the potato is widespread yet little research has been done on the problem and there is need for information based on field trials. Estimates of the losses caused vary considerably and appear to be founded on the damage to young shoots and not on crop yields.

The prevalence of black scurf on potatoes grown near Warburton, Cheshire, suggested that this was a suitable area in which to study the effect of planting clean and contaminated seed, and the relation between the amount of black scurf on the crop tubers and the dates of planting and harvesting. Although the soil is light and well drained (Table 1), the area is not an early one and is subject to late frosts.

Table 1. *Type of soil at Warburton, Cheshire*

Coarse sand	48·66	Moisture	3·14
Fine sand	21·95	Loss by solution	1·60
Silt	8·22	Organic matter	7·28
Clay	9·15	Total	100·00

The cultural, planting, and harvesting operations were carried out at the usual times for the area.

Experimental

Cultivation, manuring, planting

1941 *season.* The field had been cropped with potatoes in 1937, oats in 1938 and 1940, and Brussels sprouts in 1939. It was ploughed, cultivated and ridged, and on 26 Apr. dung (10 tons/acre) was applied and the ridges were split back to cover the dung. The seed was planted 4 in. deep on top of the dung; the rows (each was $\frac{1}{484}$ acre) were 27 in. apart, contained thirty seed tubers 16 in. apart, and were so distributed as to minimize the effect of any irregularity in the occurrence of *Corticium* in the soil. Unless otherwise stated each plot consisted of two rows, and paths, 4½ ft. wide, separated the plots. On 17 June each row received 1 lb. of a potato fertilizer and on 26 June the final ridging was carried out. The *p*H of the soil in April was 5·5.

1942 *season.* The field had been cropped with potatoes in 1939, oats in 1940, and grass in 1941. The grass was skimmed over in Nov., ground limestone (5 tons/acre) applied in Jan. and the field ploughed, cultivated and ridged in Apr. No dung was used. The seed was planted 4 in. deep along the ridges; the rows were 27 in. apart and contained twenty-five tubers 12 in. apart. Two-row plots, surrounded by 4½ ft. paths, were used. On 12 June each row received 1 lb. of a potato fertilizer and on 18 June the rows were ridged for the last time. The *p*H of the soil in May was 5·2.

Variety and seed

Arran Banner was used (except in certain trials) because it is popular in the area and because black scurf is easily seen on the white skin. Scotch seed

was used in 1941; in 1942 seed saved from the 1941 trials was planted. In any one trial similar-sized seed was used; seed size (2 oz.) was selected, but occasionally (as noted in the text) large seed (3–4 oz.) was chosen. Unless otherwise stated the seed was unsprouted; where sprouts were present they were only ½–¾ in. long and none was removed before planting. Special care was taken to avoid 'blind' tubers; in 1941, fifty-three suspected 'blind' tubers were kept under observation and fifteen failed to produce shoots. The terms 'clean seed', 'lightly contaminated', and 'heavily contaminated', are defined below; the last class was planted in trials where contaminated seed was used, and no account was taken of the position of the sclerotia relative to the sprouts. Plant counts were made during the season; a miss was recorded when no shoots were visible above soil level; plants with a few or small shoots were noted.

Inoculation. In March of each year cultures were made from sclerotia taken from tubers. Subcultures were prepared in 4 in. Petri dishes, and at planting time each culture was macerated with a little water and added to 1 pint of water; this hyphal suspension was used to inoculate two rows of seed tubers. The stirred suspension was poured over the seed after the seed had been set in the rows and immediately before it was covered with soil. Owing to the method of planting on ridges, the wide rows, and the paths surrounding the plots, there was little risk of the fungus spreading to adjacent rows or plots.

Harvesting and examination

The crop was lifted by hand and, except for a few plots, at the usual time for the area. The 'chats' formed only a small proportion of the yield; in the tables the total yields, including 'chats', are recorded, and in considering these it should be remembered that only small plots surrounded by paths were used.

The potatoes were washed, examined for black scurf, and classified as follows:

Clean. No sclerotia visible to the naked eye.

Light. Two or three definite sclerotia (2–3 mm. across) or several smaller ones.

Heavy. Sclerotia numerous and obvious. It was easy to select these tubers, and the few border-line cases between the 'light' and 'heavy' classes did not affect the results. The tables include only heavily contaminated tubers, which are expressed as percentages of the total number of tubers examined: evidence suggests that lightly contaminated tubers are equivalent to clean seed in practice.

Soil moisture and temperature

The trial area was divided into four sections; in each section several samples were taken 4 in. deep along the ridges, mixed together, and a composite sample withdrawn. The maximum and minimum soil-moisture values obtained at each sampling from May to Sept., and expressed as percentages of the wet samples, are given in Table 2. The soil temperatures (4 in. deep) are taken from the weekly mean figures recorded in the Warburton Registers of the Agricultural Meteorological Scheme.

The soil moisture was greater in 1942 but the soil was never wet for long; 1941 was a dry season for the area. The temperatures for the 2 years are difficult to compare; for 4 weeks in May and 3 in Sept. they were lower in 1941; for 5 weeks in July they were higher in 1941; in June they were almost equal and in Sept. 1942 big variations occurred.

TABLE 2. *Moisture and temperature of soil in experimental plots*

	Soil moisture				Soil temperature (° F.)				
	1941		1942			1941		1942	
No. weeks after planting	Min.	Max.	Min.	Max.	Month	Min.	Max.	Min.	Max.
1	13·9	15·7	26·8	28·2	May	43·4	50·2	45·7	52·6
3	14·6	17·3	25·5	27·4	June	52·8	60·9	52·7	59·9
5	13·6	21·9	16·2	19·6	July	58·6	63·4	57·2	60·0
8	11·7	12·5	9·4	12·4	Aug.	55·4	60·9	58·0	59·4
13	9·7	12·2	16·9	17·8	Sept.	54·9	58·4	49·1	61·7
16	13·4	17·7	18·4	24·2					
21	12·1	13·7	17·3	21·2					

Effect of planting clean and contaminated seed

1941 *season.* On 28 Apr. two rows of each of the following were planted: clean seed, contaminated seed treated in Feb., and contaminated seed untreated. Preliminary seed treatment tests were made on sclerotia 3–4 mm. across; contaminated tubers were washed, immersed for 30 sec., dried overnight and the sclerotia plated. The most effective fungicide tested, a proprietary organo-mercury preparation, which killed forty out of sixty-five sclerotia, was used in the field trials. In field tests the fungicide was less effective than in laboratory experiments. On 21 July only five misses were noted, and in Sept. the spore stage of *Corticium* was prevalent in each row. The plots were harvested on 6 Oct.; the tubers, 1356, were examined and the percentage of black scurf per row was: clean seed, 8 and 20; contaminated

seed treated, 25 and 44; contaminated seed untreated, 49 and 52.

1942 *season. Trial* 1. On 7 May three rows of each of the following were planted: clean seed, clean seed treated, contaminated seed, contaminated seed treated. Treatment was given in Dec. 1941 and at planting time the sprouts were ½ in. long. On 13 June only two misses were recorded. By mid-Aug. the haulms began to ripen and on 18 Sept. they were dead; the crop was dug on 1 Oct. and the tubers, 1987, were examined. The results are summarized in Table 3.

Trial 2. On 8 May two rows of clean seed and two of contaminated seed were planted of each of the varieties Arran Banner, Kerr's Pink (two vigorous varieties), Majestic and King Edward (two less vigorous varieties). Large seed with sprouts ½–¾ in. long were used. On 3 July only two misses were recorded. The plots were dug after the haulms had died; Kerr's Pink on 15 Oct. and the remainder on 23 Sept.; altogether 2709 tubers were examined. The results for each row are given in Table 4, and the same row order is maintained in the black scurf and yield columns.

TABLE 3. *Effect of planting clean and contaminated seed on black scurf and crop yield*

Seed	Clean		Clean treated		Contaminated		Contaminated treated	
Row no.	Black scurf	Yield (lb.)	Black scurf	Yield (lb.)	Black scurf	Yield (lb.)	Black scurf	Yield (lb.)
1	6	58	3	56	35	54	27	59
2	22	54	20	49	57	52	53	53
3	62	49	52	50	76	45	64	50
Total yield*		161		155		151		162

* These represent yields of from 17·6 to 18·6 tons/acre.

TABLE 4. *Effect of planting clean and contaminated seed on black scurf and crop yield; variety trials*

	Clean seed (2 rows each var.)		Contaminated seed (2 rows each var.)	
Var.	Black scurf	Yield (lb.)	Black scurf	Yield (lb.)
Arran Banner	28; 45	36; 37	38; 56	52; 39
Kerr's Pink*	26; 28	35; 32	21; 41	42; 30
Majestic	35; 43	32; 30	55; 61	30; 35
King Edward	11; 31	26; 33	42; 59	29; 29
Total yield		261		286

* Only twenty tubers, instead of twenty-five, were planted per row.

The results of the three trials recorded above show that a considerable amount of black scurf developed on the clean seed plots, but that this was less than on the contaminated seed plots. Seed treatment caused little, if any, decrease in black scurf, and all four varieties were heavily contaminated.

The yields from clean seed were no greater than those from contaminated seed, and there was no relation between the yield and the amount of black scurf on the crop tubers. In trial 1 (1942) all the rows were equally vigorous; in trial 2 (1942) the clean seed rows were more advanced on 9 June, but this difference had disappeared 2 weeks later. Premature yellowing or death of the haulms on the contaminated seed plots was not observed, and the spore-stage of *Corticium* was present on all plots in Sept. Only three wilted shoots (the term 'wilted shoots' refers to wilting of well-developed haulms caused by stem canker and not to young shoots) were found in 1942; this is surprising in view of the probable presence of much stem canker as suggested by trial 3 (see below).

Trial 3. On 8 May two rows of large clean seed and two of large contaminated seed of Arran Banner and Majestic, with shoots ½–¾ in. long, were planted alongside trial 2. From 23 to 25 June the plants were lifted and examined for stem canker and tuber formation; a few seed tubers were decayed so that it was not possible to find every primary shoot. The results are given in Table 5.

TABLE 5. *Attack on primary and growing shoots and tuber formation*

	No. primary shoots killed	Growing shoots		
Row no.		No.	No. attacked	No. tubers
1*	3 (1)‡	121 (25)	3+ 3§ (2)	179 (23)
3*	0 (0)	116 (25)	2+ 0 (2)	180 (24)
5*	1 (1)	123 (25)	12+ 0 (6)	160 (23)
7*	0 (0)	104 (25)	10+11 (9)	172 (25)
2†	38 (17)	115 (25)	37+14 (18)	23 (12)
4†	61 (22)	153 (25)	27+25 (20)	31 (9)
6†	40 (17)	118 (25)	18+31 (20)	43 (15)
8†	45 (20)	112 (25)	29+17 (20)	35 (13)

Rows 1–4 = Arran Banner; 5–8 = Majestic.

* Clean seed. † Contaminated seed.

‡ There were twenty-five plants per row; in each column the figures in brackets show the number of plants contributing to the result given.

§ The first figure denotes a small lesion (¼–½ in. long), the second a larger lesion (usually more than ¾ in. long).

Only four primary shoots were killed on the clean

seed plots, whereas 184, distributed over seventy-six plants, were killed on the contaminated seed plots. Despite this attack the plants on the latter plots had more growing shoots (all of which were several inches long) than those on the clean seed plots, and there were no misses. Examination of the growing shoots revealed that stem canker was present on 209 (111 slightly and ninety-eight severely attacked) distributed over seventy-eight of the 100 plants from contaminated seed; the number of healthy shoots was 289. In contrast to this, stem canker occurred on only forty-one shoots (twenty-seven slightly and fourteen severely attacked) distributed over nineteen of the 100 plants from the clean seed; there were 423 healthy shoots.

Young tubers (minimum size $\frac{1}{4}$ in. diam.) were present on ninety-five plants on the clean seed plots, the total being 691, whereas tubers were present on only forty-nine plants on the contaminated seed plots, the total being 232. On the former plots nineteen diseased stolons were found and on the latter thirty-six.

that fewer tubers, excluding chats, were produced on plants raised from contaminated seed, a result which suggests that black scurf may act as a pruning agent, reducing the number of shoots and stolons, and giving rise to fewer but larger tubers. Further trials are needed to confirm this observation. It would be interesting to repeat the experiments using seed with the number of sprouts reduced to two or three.

Relation between date of lifting and black scurf

1941 *season.* The trial consisted of six plots (three inoculated and three not inoculated), each of three rows planted on 28 Apr. with clean seed. At 3-week intervals beginning 1 Sept. six rows (four outer and two inner), one from each plot, were dug and examined. Altogether 4062 tubers were inspected.

1942 *season.* The trial consisted of eighteen two-row plots (nine inoculated and nine not inoculated), planted 6 May with clean seed, having sprouts $\frac{1}{2}$ in. long. At 3-week intervals beginning 31 Aug. six plots (three inoculated and three not inoculated) were dug and examined. Altogether 6646 tubers were inspected.

TABLE 6. *Showing relation between date of lifting, black scurf, and yield*

	Black scurf						Yield (lb.)						
	Inoc. rows			Non-inoc. rows			Inoc. rows			Non-inoc. rows			
Date dug													Total
1. ix. 41	0	0	0	0	0	0	48	59	67	45	52	67	338
22. ix. 41	5	8	23	5	8	21	80	95	69	85	78	66	473
13. x. 41	17	56	56	15	23	38	93	68	102	82	63	101	509*
31. viii. 42	8	13	22	13	20	20	45	49	37	45	40	39	514
	26	26	27	20	21	27	44	45	45	40	41	44	
18. ix. 42	24	31	34	5	11	16	52	52	45	51	56	54	605
	37	38	47	21	22	34	55	46	50	51	40	53	
8. x. 42	31	34	38	26	39	49	56	51	45	45	50	51	602†
	47	59	62	53	58	73	46	51	53	55	52	47	

* 18·3 tons/acre.

† 17·3 tons/acre.

The results show that despite favourable conditions for the disease and the prevalence of *Corticium* in the soil, the attack on the clean seed plots was negligible compared with that on the other plots, a result which indicates that the disease on the young shoots and the check to tuber formation were caused mainly by the fungus on the seed tubers and not by that in the soil.

From these results it is probably safe to assume that the plants raised from contaminated seed in trials 1 and 2 were also severely attacked. Examination of such a crop early in the season might lead to the conclusion that black scurf is a serious disease. Yet the recovery was almost complete in that few misses occurred, there was no premature yellowing or death of the haulms, and the yields were satisfactory. The fact that so few misses occurred throws doubt on *Corticium* as the cause of many permanent misses under farm conditions, for in these trials all the seed was heavily contaminated.

A study of the results of trials 1 and 2 revealed

The results are summarized in Table 6; the amount of black scurf is given row by row and the same row order is maintained in the yield columns.

Least black scurf occurred on the earliest dug plots and most on the latest dug. In 1942 the attack was earlier and more severe; in this year the earliest dug crops were heavily contaminated but in 1941 they were almost clean. The heavier attack in 1942 may be due to such factors as lack of dung, higher soil moisture, and heavier *Corticium* infection of the soil, but the data are insufficient to permit conclusions.

Despite the use of clean seed a high percentage of the later dug crops was contaminated, which, together with the frequent incidence of the spore stage of *Corticium*, shows that the fungus was prevalent in the soil. This may explain why no appreciable difference in disease development occurred between the inoculated and non-inoculated plots.

Good yields were obtained each year; the increase was significant from the 1st to the 3rd week in Sept., thereafter it was small in 1941 and nil in 1942. In 1942 the crops died earlier probably owing to lack of dung. No relation was apparent between the yield and black scurf contamination. In the three-row plots of 1941 the yields from the middle rows were lower than those from the outer rows; for this reason two-row plots were used in 1942.

In addition to the results given above the following observations were made. In 1941 the number of misses on plots 1–6 (180 seed tubers/plot) on 23 June, 3 days before the final ridging, was 9, 2, 7, 1, 5 and 6 respectively; of these, twenty-one were lifted and examined, and in every case the primary shoots had been killed and fresh shoots were growing from these shoots or direct from the seed tuber. None of the fresh shoots had yet appeared above soil level and hence the plants had been recorded as misses. The plants were replaced after examination and on 26 July only six misses were recorded in the whole trial. In 1942 the stand was very regular and only one miss was recorded on 3 July. In both years wilted shoots were rare and the spore stage of *Corticium* was prevalent towards the end of the season.

Effect of late planting on black scurf

In 1941, four plots (two inoculated and two not inoculated) were planted on 28 Apr. with clean unsprouted seed; an equal number of similar plots was planted on 4 June, the seed having sprouts ½ in. long. The trial was repeated in 1942 when the planting dates were 6 May and 12 June respectively; the sprouts at planting time were ½ in. long in May and 1 in. or more in June. The plots were harvested together after the haulms had died. In each year 37 days elapsed between early and late planting and 22 weeks from early planting to lifting. The number of tubers examined for black scurf was 4333 in 1941 and 2095 in 1942.

The results showed that although black scurf was less on the late-planted plots it was heavy on all plots; there was no apparent difference between inoculated and non-inoculated rows. The yields from the late-planted plots, which averaged 17 tons/acre in 1941 and 14½ tons/acre in 1942, equalled those from the early-planted.

Variety trials

1941 *season.* On 29 Apr. three rows of each of the varieties Arran Banner, Kerr's Pink, Majestic and King Edward were planted with clean, large, seed just beginning to sprout. One row of each variety was inoculated at planting time. On 21 July only four misses were recorded. On 13 Oct. the plots were dug and the tubers, 2541, examined. Each variety was heavily attacked in the inoculated and non-inoculated rows.

1942 *season.* The same varieties were tested in trials 2 and 3 (1942) and, as shown in Table 4, they were all heavily contaminated and all recovered well from the probable early attacks on the young shoots.

Discussion

In all the field trials, including clean seed trials, a high proportion of the crop was heavily contaminated and the spore stage of *Corticium* appeared frequently; in 1942 the young plants were severely attacked and black scurf appeared earlier and was worse than in 1941. These facts indicate that *Corticium* was prevalent in the soil and that in 1942, especially, conditions were favourable for the disease.

Under these conditions the use of clean seed resulted in no apparent check to growth, only a slight attack on the young shoots, and in a satisfactory crop yield. On the contaminated seed plots, however, the young plants were severely injured and tuber formation was checked. Thus, the attack was caused almost entirely by the fungus on the seed tubers and not by that in the soil, a result which might suggest that black scurf is harmful on seed potatoes, and that it is advantageous to plant clean seed even in infected soil. This view is not supported by the crop yields, which were satisfactory and were no lower from contaminated seed than from clean seed. It may be noted that all the former seed was heavily contaminated, a condition not obtaining in ordinary farm practice. It might be thought that higher yields would develop in soils less heavily infected with *Corticium*, but this is unlikely in view of the small amount of injury caused by the fungus in the soil; furthermore, it is doubtful if the question arises in practice, since there is no reason to believe that the soil at Warburton is more heavily infected than that in other potato areas where short rotations are practised.

Recovery from early attacks was noted by Edwards (1929) in pot experiments and by Miles (1930) in field trials. In the 1942 trials recovery was not assisted by liberal manuring or cultural means, since the seed was not specially sprouted, dung was omitted (there appears to be general agreement in the literature that less injury occurs where dung is used) and very little fertilizer was applied. It may have been helped by the soil type (light and well drained), by planting whole tubers from which no sprouts had been removed, and by the better growing conditions prevailing when the new shoots were formed to replace those killed by *Corticium*. The large seed in trial 2 (p. 223) may also have helped recovery, but this does not hold for trial 1 (p. 223) where seed-size tubers were used. The planting of contaminated seed may, in effect, be equivalent to late planting, and it is interesting to note that the yields from the early- and late-planted plots were equal.

Further work is needed before definite conclusions

can be drawn, but the evidence so far obtained supports the view that in this country *Corticium Solani* in the soil or on the seed causes little, if any, loss under ordinary farm conditions and that recommendations involving the selection of clean seed, seed treatment, and attempts to raise clean seed are of doubtful value. Clean seed may be secured by early lifting in certain seasons but is likely to be attended by serious loss in yield. These conclusions may not apply to heavy, cold soils, or where the sprouts have been reduced to two or three before planting. Pethybridge (1911) and Davidson are of the opinion that the disease causes little loss in Ireland.

Thanks are due to the Agricultural Research Council who financed the work in 1941. For the *p*H values, moisture determinations and mechanical analysis of the soil given in this paper I am indebted to Dr R. Stewart, Agricultural Advisory Department, Manchester University; for the soil temperatures, to Dr M. Cohen of the same Department.

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Investigation into the production of bacteriostatic substances by fungi

II. A method for estimating the potency and specificity of the substances produced

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(With Plate 4)

A method is suggested for estimating the degree of potency and, to some extent, the specificity of bacteriostatic substances produced by fungi. It is based on the examination of several hundred fungi over a period of 2 years. It consists essentially of the placing of a few drops of the substance to be tested in the centre of a plate of bacteria-incorporated agar with the consequent production of a zone of inhibition which varies in width in proportion to the concentration of the bacteriostatic substance. The test is made against two representative types of bacteria—*Bact. coli* and *Staph. aureus*. The permissible technical latitude in the application of the test has been summarized. By using a standard inhibitor (mercuric chloride) the accuracy of the method has been statistically proved. This 'zonation' method has been compared with the standard 'dilution' method and close correlation has been established.

Wilkins & Harris (1942) showed the variability of different fungi in the production of anti-bacterial substances and stated that the degree of inhibition produced by any one positive fungus is also variable. This variability appears to be influenced mainly by the cultural conditions under which the fungus is grown. Work is now being done on the effect of cultural conditions on the production of bacteriostatic substances, but, in order that the results obtained may be regarded as varying in relation to the substance being tested rather than in relation to the method of testing, it is necessary to establish a testing method which will give consistent results under standard conditions. An attempt has been made to standardize a method so that the potency and specificity of bacteriostatic substances can be estimated qualitatively and quantitatively, and so that the results of different experiments at different times by different observers shall be comparable.

EXPERIMENTAL

Methods in general

The methods tried fall into two main classes—'dilution' and 'zonation'. The former are standard bacteriological technique, the latter are simpler and, if correlated with the former, as informative.

One of the simplest of the zonation methods is to grow the fungus to be tested on an agar plate and, when the colony is about 1 in. diam., to flood the plate with a suspension of the bacteria to be inhibited and incubate at 37° C. Any inhibitory substance produced by the fungus diffuses into the agar and there is a clear bacteria-free zone round the edge of the colony (Pl. 4, fig. 1). This method is useful for indicating a positive or negative result but is less generally satisfactory than methods described later. Another simple positive/negative test can be effected by pouring plates of nutrient agar incorporated with a bacterial suspension and, when cool, putting a few drops of the substance to be tested on the surface of the agar. A positive result is indicated by a bacteria-free area round the site of the drops. This test was mentioned by Lafar (1910).

A more satisfactory method is to grow the fungus on a liquid-culture medium in which inhibitory substances, if produced, will be present. Nutrient agar incorporated with bacteria is poured into plates and, when cold, a glass ring-cell with ground edge is placed on the surface of the agar: a small quantity of the culture liquid is placed in the ring-cell. The inhibitory substances diffuse into the agar during the subsequent incubation at 37° C. and produce a bacteria-free circular zone round the ring-cell. The diameter of this zone will be an indication of the relative degree of inhibition. The 'cylinder' method, as used by the workers at the Oxford School of Pathology (Abraham *et al.* 1941), is somewhat similar but more satisfactory. Plain nutrient agar is poured into plates and, when cool, flooded with a suspension of bacteria which is then dried off. A small porcelain cylinder is warmed and its base pushed slightly into the surface of the agar. The substance to be tested is placed in the cylinder and the zone of inhibition is produced round the cylinder.

The present method

After considerable experimentation, the following method has been adopted in this laboratory since July 1941. As results in subsequent publications will be expressed in terms of this method it is desirable to describe it in detail. The figures in brackets refer to notes which indicate the degree of technical latitude permissible at each stage.

Make up nutrient dextrose agar as follows: Lab. Lemco 5 g., peptone 5 g.; mix together in about 100 ml. warm distilled water and boil vigorously for 10 min. Make up to 1000 ml. with distilled water. Add 20 g. powdered agar (1). Adjust to *p*H 7·0 (2). Steam for 1 hr. (3). Add 5 g. dextrose (4), and readjust to *p*H 7·4 (5). Put 250 ml. into each of four 500 ml. flasks. Autoclave for 15 min. at 126° C. (6). Cool to 50° C. (7). Add 12·5 ml. of a 24 hr. suspension of the required bacterium, i.e. in the proportion of 1 ml. suspension/20 ml. of the medium (8). The suspension is made by adding a loopful of a 24 hr. suspension of bacteria (9), to a tube containing 12·5 ml. of nutrient broth made up exactly as the agar but steamed for 30 min. only. Agitate to distribute the bacteria and pour into cool plates in 20 ml. quantities (10): this is most conveniently done by using an automatic measuring apparatus (11), which is fitted to each flask. At the end of 15 min. (12), when the agar has gelled, with a sterile cork borer (no. 6) cut a hole (or holes) 11 mm. diam. (13) in the agar. With a sterile pipette put four drops (14), i.e. approximately 0·1 ml. of the fungus culture liquid to be tested, into the hole and incubate at 37° C. (15) for about 18 hr. (16). Measure the diameter of the area of inhibition (17).

Notes

(1) The addition of either 10 or 30 g. of agar makes no difference to the result, but 30 g. is unnecessarily extravagant, while 10 g. makes the agar more difficult to cut later.

(2) This should be approximately correct or the amount of acid added later will be variable and so affect the composition of the medium.

(3) Steaming for ½ hr. is not satisfactory, but steaming for longer than normal, e.g. 1½ hr., makes no difference.

(4) The dextrose is not essential but tends to give a better film and a sharper edge to the area of inhibition.

(5) The reaction should not be less than *p*H 7·0 but may be as much as *p*H 7·6 with no serious effect.

(6) Increasing the autoclaving time to 45 min. makes no difference.

(7) This is important. If cooled below 50° C. the agar is likely to gel before the pouring can be completed, and if the temperature is higher the viability of the bacteria is impaired. It can be kept in a 50° C. incubator but not longer than 15 min. or the film is granular.

(8) Using a suspension of half this concentration causes the film to be less satisfactory, but double the concentration makes no appreciable difference.

(9) Inoculation from a 24 hr. slant gives a slightly low result, while inoculation from a 48 hr. suspension tends to give a slightly high one. In neither case, however, is the deviation serious.

(10) Using 10 or 30 ml. quantities, which alters the depth of the agar in the plate, makes no difference to the result, but 30 ml. is less economical and 10 ml. gives a thin agar which is difficult to cut accurately and which is unsatisfactorily shallow.

(11) Obtained from A. Gallenkamp and Co., Ltd., London.

(12) The plates can be left at laboratory temperature (about 17–18° C.) for any time up to 6 hr. A higher temperature allows the bacteria to develop, and keeping in a refrigerator impairs the viability of the bacteria and gives erroneous results. It was hoped to find a temperature at which plates could be kept for some time. Certain plates were incubated at −20, −10, −6, −3, 0, 5, 10, 15 and 20° C. for a week and a test was then made against two different types of bacteria. Without stating details, it may be said that in the case of both bacteria, only a temperature between 5 and 10° C. is satisfactory for keeping the plates, and even then it is not to be recommended for more than a day or two.

(13) Any diameter of hole from 8 to 12·5 mm. will do equally well, but 11 mm. is the most convenient size for the number of drops used here.

(14) Two drops are not sufficient, but 3, 4 or 5 drops are satisfactory: 6 drops makes the hole too full and the liquid is likely to spill over the surface.

(15) A range of 3° C. on either side of 37° C. makes no appreciable difference to the result.

(16) The area of inhibition is faintly visible after 6 hr. but has not then reached its final size: 18 hr. is a convenient overnight period and the zone is then stable.

(17) The diameter of the area of inhibition is measured, as giving a lower percentage error, but the final result is expressed as the width of the zone from the edge of the hole to the perimeter of the circle.

The above method attempts to standardize the conditions so that any variation in the area of inhibition must necessarily be due to the culture liquid which is being tested. In order to prove this to be the case the following experiments were done with 0·5% mercuric chloride as standard inhibitor. This concentration gave a zone of inhibition approximately equal to that given by a reasonably good fungus extract. The figures obtained were constant, but with the object of producing even more conclusive evidence of constancy, the mercuric chloride solution was tested against *Bact. coli* and *Staph. aureus* in ten experiments each of ten tests. The experiments were done with different batches of media, different suspensions and by three people working independently. In order to show the consistency of the results, those of the first *five* experiments are given in Table 1.

The figures illustrate the accuracy of the method. Analysis shows that in the case of a given set taken at random, the standard deviation, using the formula $\sigma = \sqrt{\frac{\Sigma(v^2)}{n-1}}$ is with *Bact. coli* ±0·16 and with *Staph. aureus* ±0·26. By taking the deviation of the ten sets of *means* the standard deviation of a single observation is with *Bact. coli* ±0·3 which is 4·3% of the mean, while with *Staph. aureus* it is ±0·16 or 4·3% of the mean. As there is no reason to expect any greater degree of deviation against *Bact. coli* than against *Staph. aureus*, all the means can be taken together, and in this case, with eighteen degrees of freedom, the percentage error of the mean is only 2·6%.

TABLE 1. *Deviation of mercuric chloride test*

Exp.	1		2		3		4		5	
Organism ...	*Bact. coli*	*Staph. aureus*	*Bact. coli*	*Staph. aureus*	*Bact. coli*	*Staph. aureus*	*Bact. coli*	*Staph. aureus*	*Bact. coli*	*Staph. aureus*
Width of zone of inhibition (in mm.)	7·0	11·5	7·5	12·0	7·0	11·5	6·5	11·0	7·0	11·5
	7·5	12·0	7·0	12·0	7·0	11·0	7·0	11·5	6·5	11·5
	7·0	12·0	7·5	11·5	7·0	11·5	6·5	11·0	6·5	11·5
	7·0	11·5	7·0	11·0	7·5	11·5	7·0	11·5	7·0	11·0
	7·0	11·5	7·0	11·5	7·0	11·0	7·0	11·5	6·5	10·5
	7·0	11·5	7·0	11·5	7·0	11·5	6·5	11·0	6·5	11·5
	7·0	11·5	7·5	11·5	7·0	11·0	6·5	11·0	6·5	11·5
	7·0	11·5	7·5	11·5	7·0	11·0	7·0	11·5	7·0	11·5
	7·0	11·5	7·5	11·5	7·0	11·5	7·0	11·0	7·0	11·5
	7·0	11·5	7·5	12·0	7·0	11·5	6·5	11·5	7·0	12·0
Mean width	7·05	11·6	7·3	11·6	7·05	11·3	6·8	11·25	6·75	11·3

Comparison between 'zonation' and 'dilution' methods

The effectiveness of inhibiting substances is often expressed in terms of the degree of dilution at which they are still inhibitory. We have attempted a correlation between the size of the zone as obtained by the zonation method and the degree of dilution effect.

The 'dilution' part of the experiment was done by using two series each of sixteen tubes. In tube 1 in each series was put 5 ml. of a 0·5% solution of mercuric chloride in nutrient broth. In each successive tube the mercuric chloride was diluted by half with nutrient broth, so that tube 1 contained a 1/200, tube 2 a 1/400, and tube 16 approximately a 1/6,000,000 concentration of mercuric chloride. To each tube in the first series was added one drop of a 24 hr. suspension of *Bact. coli*, and to each tube in the second series one drop of a similar suspension of *Staph. aureus*. The tubes were incubated for 48 hr. and the results are shown in Table 2. Visible bacterial growth (i.e. no inhibition) is indicated by +, no bacterial growth (i.e. inhibition) is indicated by −.

Fig. 1

Fig. 2

WILKINS AND HARRIS—INVESTIGATION INTO THE PRODUCTION OF BACTERIOSTATIC SUBSTANCES BY FUNGI

The 'zonation' part of the experiment comprised two series each of sixteen plates prepared as previously described. The first series had *Bact. coli* and the second series *Staph. aureus* incorporated in the agar. Concentrations of mercuric chloride solution in distilled water were added as in dilution experiment in a 1/2 serial dilution. The results are expressed as the width of the zone of inhibition in mm. The two methods were therefore reasonably comparable. The results are shown in Table 2.

When the zonation figures are plotted, concentration of mercuric chloride against width of zone, the result is approximately a straight line in both cases, and it seems that an increase of something between 0·5 and 1·0 mm. in zone width is equivalent to a doubling of the concentration. This is also true of most fungus extracts which have been similarly tested.

The above method has proved most reliable in practice, as it is simple, rapid and consistent. Moreover, as shown in Pl. 4, fig. 2, the zones are sharply defined, regular in outline and easily and accurately measurable. A similar method has been used by Fleming (1942) for testing the bacteriostatic influence of penicillin, lysozome and other chemical substances. The 'sealing' of the bottom of the hole as advocated by Fleming has proved unnecessary.

TABLE 2. *Comparison of 'zonation' and 'dilution' methods*

	Dilution of mercuric chloride														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
	Bact. coli															
Dilution	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+
Zonation	7·5	6·5	5·5	5·0	4·0	3·0	2·5	1·5	1·0	−	−	−	−	−	−	−
	Staph. aureus															
Dilution	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+
Zonation	11·5	9·5	8·5	7·5	6·5	5·5	4·5	3·5	3·0	2·5	1·5	0·5	−	−	−	−

In the case of both bacteria, as the concentration of mercuric chloride is progressively reduced, a stage is reached where, by the dilution method, turbidity indicates lack of inhibition and by the zonation method no zone is produced. In the above experiments the correspondence between inhibition as indicated by the dilution and zonation methods is quite exact. This is not the case with all inhibitors, and even here the end-point varies with the type of bacteria. With *Bact. coli* the last zone is produced at what corresponds to a dilution of approximately 1/100,000 while with *Staph. aureus* the last effective dilution appears to be approximately 1/400,000. In the dilution method the delineation of the inhibition was sharply defined as on plating out the non-turbid tubes no growth was obtained.

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EXPLANATION OF PLATE 4

Fig. 1. Showing zone of approx. 22 mm. produced against *Staphylococcus* by a growing culture of *Penicillium claviforme*.

Fig. 2. Showing zone of approx. 6 mm. produced against *Staphylococcus* by an extract of a fungus sporophore.

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Direct microscopical observations upon the rumen population of the ox

I. Qualitative characteristics of the rumen population

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The rumen population of the ox comprises three major groups of micro-organisms: (*a*) Protozoa, (*b*) iodophile micro-organisms, (*c*) aniodophile micro-organisms. The iodophile species comprise macro and micro forms. The forms include several new species and genera which are described in the paper. A fixed and a free iodophile population are discriminated, the former constantly attached to starch grains and vegetable fragments, the latter in suspension in the rumen liquid. The rumen population was qualitatively independent of the range of diet and of the breed of animal examined. Protozoa play no part in the decomposition of cellulose but digest starch. Decomposition of both starch and cellulose is accomplished by the fixed iodophile microflora which, in the process, synthesize iodophile polysaccharide. During passage through the alimentary canal the rumen population is eliminated, the Protozoa by peptic and tryptic digestion and the iodophile microflora by (*a*) digestion by Protozoa, (*b*) autolysis, (*c*) action of digestive enzymes. The products of microbial synthesis are held to be assimilated by the host animal.

Introduction

It is generally accepted that the microflora and microfauna of the rumen make an important functional contribution to the processes of digestion and assimilation in ruminant herbivora. In regard to the nature and extent of this contribution divergent opinions are held and it seems clear that a just assessment of conflicting claims and opinions will be facilitated by an accurate characterization of the indigenous micro-organisms of the rumen as members of a complex functional association. In the absence of such information a standard of normality is lacking to which variations can be referred, whether these are encountered in the examination of post-mortem material, of fistular samples, or of rumen contents incubated *in vitro* under controlled experimental conditions. Two methods are available in an enquiry directed towards this end:

(1) The isolation in pure or mixed culture of the component micro-organisms of the association.

(2) The direct microscopical observation in film preparations, both of the total microfauna and flora and of their relations to the microscopically accessible components of the substrate.

The first method appears to offer greater facilities for the collection of accurate and reliable data than the second and, no doubt for this reason, has been widely adopted.

The results obtained by its use have, however, proved inconsistent in practice, a situation to which several factors contribute. Thus, first, many of the micro-organisms demonstrable microscopically in fresh material can only be grown *in vitro*, if at all, with great difficulty and so usually fail to appear on the culture media employed. Secondly, casual passengers through the alimentary canal normally ingested with the food, and which may originate from the soil, water, or atmosphere, may be far less exacting in their requirements and are, therefore, represented by an impressive number of colonies. Finally, even where the growth requirements of the less tractable indigenous species can be ascertained, neither the composition of the media nor the conditions of cultivation are such as lend themselves readily to a general investigation of the total microflora and microfauna. Until such difficulties can be overcome it seems unlikely that a comprehensive picture of the microbial associations of the gastro-intestinal tract will be obtained by pure cultural methods. Further progress in the field of rumen microbiology, therefore, can only be expected if full use is made of the method of direct microscopical examination. It has been shown (Baker, 1939) that such methods are not necessarily confined to a bare enumeration of species but can also be employed to determine the changes taking place in the starch and cellulosic components of the materials fed. In this paper an account is given of the microflora and microfauna of the rumen of the ox as elucidated by these procedures. Where the results obtained are similar to those already described for other animals they are summarized briefly.

Sources of materials

The rumen contents examined were obtained either from the local slaughterhouse material of Messrs Colebrook, Guildford, or from a fistula animal at the experimental farm of the Hannah Dairy Research Institute, Kirkhill, Ayr. Post-mortem material was removed through a large incision made in the rumen immediately after the death of the animal. Several

pounds of material were collected on each occasion, including both solid and liquid fractions, and fixed without delay in jars of 10% commercial formalin. Penetration was ensured by vigorous stirring and shaking. Fistula material was collected at Kirkhill in sample bottles each containing 90 ml. of 10% formalin, 10 ml. of rumen liquor was run into each bottle with a pipette and the mixture shaken. The samples collected were posted to Guildford for microscopic examination.

The pedigree of the fistula steer as far as records go allow it to be described as a Friesian-Ayrshire cross. The samples were taken over the period 3 Mar. 1940–30 June 1942. The materials fed during this period included hay, together with blood meal, urea and cereal rations. About 10 lb. of hay were fed per day. The composition of the rations was as follows:

(1) *Blood-meal ration.* Oats 2¾ lb., bran 2 lb., starch ⅔ lb., molasses ⅔ lb., blood meal ⅓ lb.

(2) *Urea ration.* Oats 2¾ lb., bran 2 lb., starch 1 lb., molasses ⅔ lb., urea 40 g. (the urea was dissolved in the molasses).

(3) *Cereal ration.* Oats 4⅔ lb., bran 2 lb., molasses ⅔ lb.

(4) A further ration fed was identical with (2) except that urea was omitted.

Samples were also taken from the animal when it was at grass. During this period it received a urea ration.

METHODS

Dry-film preparations for bacteria and smaller micro-organisms

Films spread on cover-glasses were fixed by heat or, preferably, with ethyl or methyl alcohol, with or without addition of formalin, and were stained by various methods according to the end specifically in view. Carbol-fuchsin, methylene-blue and the Romanowsky stains were suitable for routine examinations. The last included Jenner's, Leishman's, Giemsa's and Pappenheim's combinations and was employed either directly or according to the panoptic modification (Lee, 1937) using buffered water for differentiation and as diluent. Gram preparations were made according to the standard procedure and by both Weigert's (anilin xylol) and Claudius's (picric cloves) modifications. Negative mounts in nigrosin were useful for general purposes and for the demonstration of capsules, spirochaetes, etc. For the latter Loeffler's flagella stain and Fontana's silver method were also employed. Neisser's method was used for the demonstration of volutin granules and Moeller's procedure for the study of spores (Eyre, 1930).

Iodine preparations

The examination of freshly made iodine preparations was an integral part of the routine procedure. Gram's iodine was employed with or without the addition of chloral or glycerin as clearing agents (Baker & Martin, 1937*b*).

Protozoological methods

A detailed cytological characterization of the rumen microfauna was not attempted. Structural features were considered only in so far as they contributed to taxonomic identification of the species present or to a better understanding of their functional capacities and relationships. Dry films were useless in the examination of these organisms. In addition to iodine, picrocarmine and acetic iodine-green were employed, particularly for the rapid demonstration of nuclear structures. For more detailed examination and as type slides for permanent reference Mayer's haemalum and Heidenhain's iron haematoxylin were used. Preparations, after staining, were graded through alcohols into mixtures of Gurr's medium and alcohol of gradually increasing concentration.

Microchemical examination of the substrate

Many of the cell-wall components of the vegetable structures could readily be identified, and changes occurring in them demonstrated *in situ* by the use of microchemical reactions. Chlorzinciodine and Zimmerman's reagent were used for the detection of free cellulose, Sudan III for the demonstration of cutin, phloroglucinol or aniline sulphate for lignin, and ruthenium red for pectic substance: where other reagents were employed, they are indicated.

Use of polarized light in the investigation of cell-wall components of substrate

It has been demonstrated that the changes which accompany the microbiological disintegration of cellulose structures can be followed *in situ* by the use of the polarizing microscope. Cellulose is strongly birefringent and, under polarized light, regions in process of disintegration are sharply outlined as dark areas upon a brilliantly illuminated background: an intense source of light is necessary. The preparations are made up in Stephenson's medium (Lee, 1937) which is a saturated aqueous solution of mercuric and potassium iodides, R.I. 1·67. Iodine is added for the demonstration of iodophile micro-organisms.

FRACTIONATION OF RUMEN CONTENTS

The heterogeneity of the rumen contents was appa rent to the naked eye. On diluting the contents with, for example, ×10 their bulk of 10% commercial formalin, and agitating gently with an electric stirrer for a period of ½ hr., and then straining through muslin on a Buchner plate, the bulk of the coarse vegetable material (fraction A) was retained and an opaque suspension filtered off. This suspension, on standing for 1 hr., deposited a sediment (fraction B) and, within a second hour, a further sediment (fraction C). The fluid successively decanted from these

fractions yielded after 12 hr. a fine deposit (fraction D). The liquid removed remained stable for several days but, on centrifuging, yielded a still finer sediment (fraction E). These fractions possessed, on average, distinct microbiological characteristics. Fraction B contained the rumen Protozoa and a variable proportion of small vegetable fragments. In fraction C the ratio of the components was reversed, vegetable debris preponderating. Fraction D contained the bulk of the larger iodophile micro-organisms (see p. 233). Fraction E comprised a number of smaller bacterial species, spirochaetes, etc. In samples taken from different rumen levels the proportion of solid to liquid fractions diminished progressively with the depth from which the sample was taken.

Microfauna of the rumen

The rumen microfauna which sediments from fraction B in less than 1 hr. comprises an indigenous population of Protozoa, whose total number, under normal feeding conditions, rarely falls below several hundred thousand per ml. (Mangold, 1929). The morphology and taxonomy of these organisms have been elucidated by a number of investigators (Doflein, 1928; Wenyon, 1926). The larger number of the forms indigenous to cattle are oligotrichous ciliates of the suborder Entodiniomorpha. Species of the family Ophryoscolecidae are distributed throughout the genera *Ophryoscolex*, *Diplodinium*, *Epidinium*, and *Entodinium*. The protozoan population includes, in addition to these oligotrichous types, a number of holotrichous ciliates of the suborder Stomatea, including the genera *Isotricha* and *Dasytricha*. The actual group of species present tends to remain constant for each species of ruminant (Mangold, 1929), but minor individual variations are noticeable in the rumen contents taken from different animals of the same species. Thus, in the present investigation, *Ophryoscolex purkenjeyi* was always encountered in the Guildford post-mortem material, though it was never observed in fistula samples from the Hannah Institute.

Functional activities of the rumen ciliates

The functional activities of the Protozoa, so far as they can be determined by direct microscopical observation, may conveniently be considered under the two headings of ingestion and digestion.

Ingestion

In the interior of the Protozoa starch grains, chloroplasts and other vegetable materials are commonly seen. The ingestion of these structures may in principle be largely promiscuous, though, in fact, a pronounced selectivity can be imposed upon it by the operation of purely mechanical factors. Amongst these may be noted the relationship between the actual dimensions of the particles present and (*a*) the overall dimensions and internal structure of the given protozoan species, and (*b*) the dimensions of the cytopharynx. In these respects the two major groups of Protozoa present show a marked contrast. The holotrichous species possess a small rigid cytopharynx so that, notwithstanding the considerable overall dimension attained by some species, only particles of restricted size, such as small starch grains, are ingested. In the oligotrichous species, on the contrary, not only is the cytopharynx large and elastic, but, as Trier (1926) emphasized, the interior of the endoplasm is modified so as to form a gastric sac, which affords a capacious receptacle for the materials ingested. Large starch grains, therefore, are frequently observed in the interior, even of the smaller species. In the giant Diplodinia and Ophryoscoleces, larger plant structures, such as entire plant hairs and groups of epidermal cells, are also ingested. Bacteria of all types are readily ingested by both oligotrichous and holotrichous ciliates.

Digestion

Starch and iodophile micro-organisms. That the ingested starch grains are also digested was demonstrated by Trier (1926), whose results were confirmed by Westphal (1934). Grains in all stages of digestion may be observed within the gastric sac of the oligotrichous ciliates. Similarly, the conclusion of previous observers, that digestion is accompanied by the progressive deposition of reserves of paraglycogen in the regions surrounding the gastric sac, was substantiated. In addition to starch grains the digestion of iodophile micro-organisms was observed in the material. The changes included both disintegration of the sheath and loss of the iodophile substance.

Cellulose

The capacity to digest cellulose has been attributed to the Protozoa. Even were this demonstrable their contribution to the total disintegration of cellulose occurring could only be minimal, since the bulk of the vegetable material, constituting fraction A, is made up of macroscopic tissue fragments whose dimensions preclude digestion. The evidence advanced in support of the view that cellulose is digested by Protozoa is itself unconvincing. It was easy to determine, for example, by examination in polarized light and by microchemical reactions, that the ingested particles are sometimes in an advanced state of disintegration. In such cases the nature of the changes observed (Baker, 1939) made clear that their disintegration had been occasioned, prior to ingestion, by the activities of the iodophile micro-organisms.

Chemotropic selectivity of the rumen Protozoa

In addition to the limitation on ingestion imposed by the mechanical factors instanced, there is evidence that these may be re-enforced by chemical selectivity. Dogiel (cited by Mangold, 1929) described

and illustrated the way in which large Diplodinia approach, explore, and attack the prosenchymatous 'fibres' of vegetable tissues. Such movements are clearly not of a random character and presuppose a directing stimulus. In the course of this investigation a permanent preparation demonstrating an early phase of the process described by Dogiel was secured. One portion of the fibre was deeply embedded in the gastric sac of the Protozoa, the other protruded from the cytopharynx: examination in polarized light showed that the fibre was in process of disintegration. The features presented are precisely those which uniformly attend the decomposition of prosenchymatous structures by iodophile micro-organisms (Baker & Martin, 1937*b*). Thus, it may be assumed that disintegration of the fibre had been effected prior to ingestion by the Protozoa. Similarly, it becomes reasonable to suppose that the katabolites liberated by the micro-organisms concerned in the process of decomposition afford a chemotropic stimulus to the Protozoa, directing them to the affected vegetable structures. From analogy with known data, it would seem probable that the chemotropic agents are the carbon dioxide and organic acids liberated in the process of decomposition, and it would appear, therefore, that the ingestion of particles is influenced by chemical as well as by mechanical factors. Thus, other things being equal, a preferential ingestion of material in actual process of decomposition will tend to prevail.

THE RUMEN MICROFLORA

The iodophile microflora

The rumen microflora of the ox includes a variety of micro-organisms which, since they contain substances of carbohydrate affinities (Henneberg, 1922; Simons, 1920) that give a blue reaction with iodine, are distinguished as iodophile species. Some of these organisms are suspended in the rumen liquid whereas others adhere to starch grains and to vegetable structures. The latter, which constitute the *fixed iodophile population*, are encountered throughout fractions A–D. The former, or *free iodophile population*, is chiefly concentrated in fractions D–E. Of these fractions, D includes a number of types which on account of their large dimensions may be termed macro-iodophile in contrast to the small or micro-iodophile species of fraction E. The fixed iodophile population includes both macro- and micro-iodophile types.

The presence of an abundant iodophile microflora is a distinctive feature of the rumen of ruminant and the caecum of non-ruminant herbivora (Henneberg, 1922; Baker, 1939). That the iodophile habit or character is an index of functional similarity rather than taxonomic affinity is shown by the fact that it is exhibited not only by unrelated species of bacteria but also by the colourless oscillarians native to the gastro-intestinal tract of certain animals.

The macro-iodophile species of the rumen

A. *Myxophyceae (Cyanophyceae)*

Oscillospira. This large organism ($5–20 \times 3–4\,\mu$) is closely related to the colourless oscillarian indigenous to the caecum of guinea-pigs discovered by Chaton & Pérard (1913) and redescribed by Simons (1920) as *Oscillaria caviae*. The trichome, which possesses a mucilaginous sheath, is usually slightly larger at one end than the other and contains a number of short, broad, and closely packed 'cell' segments in which the iodophile substance is pre-eminently localized. Formation of transverse walls takes place from the periphery to the centre in the manner characteristic of the Myxophyceae. Reproduction by hormogones with formation of a minimal hormogone is conspicuous. In *O. guilliermondi* spore formation occurs, but this has not yet been observed in the species here described. The organism is Gram-negative.

When properly differentiated, the cell contents stain a red violet with Romanowsky combinations, the sheath being blue. In consequence of its striking oscillarian affinities, the organism is recognizable in either dried film or iodine preparations.

B. *Schizomycetes*

In describing these organisms, all of which give a strong blue-black reaction with iodine, the genera *Amylosarcina*, *Amylospirillum*, and *Amylococcus* are, by analogy with the genus *Amylobacter*, employed for reasons of convenience. Their status is provisional. Drawings of these organisms have been published (Baker, 1942*a*).

Amylobacterium asteroides (pro tem.). The taxonomic allocation of this organism to the genus *Amylobacterium* is tentative. It is recognizable under the microscope as a rosette or star-shaped colony. The components of this structure, which comprise in the typical instance some thirty or more straight or slightly curved fusiform rodlets with acute apices, are disposed radially at regular intervals about a single centre. They are weakly Gram-positive and stain well with methylene blue and other aniline dyes. With Romanowsky combinations the contents of the central apices often become red-violet, the peripheral apices staining blue. In negative mounts in nigrosin the former regions are strongly refractive and it is in them that the maximum deposition of iodophile substance occurs. The rodlets display considerable variation in size and form, ranging from slender bacilli to navicular and clostridial types ($4–8 \times 0{\cdot}9–1{\cdot}5\,\mu$). Appearances suggest that the larger arise from the smaller forms by a progressive swelling consequent upon the deposition of iodophile substance. Such changes were shown to occur in one of the iodophile micro-organisms of the caecum of the horse (Baker & Martin, 1939).

Amylospirillum mirabile (pro tem.). The organism is $15–40\,\mu$ in length and commonly comprises a spiral

of 1·5–6 turns. Iodine preparations show that it is divided internally by transverse septa into numerous spheroidal segments of 1–2 μ average breadth. Romanowsky stains demonstrate a pinkish external sheath which is continuous with the septa and encloses the light blue-violet cell contents. Both sheath and septa are aniodophile. The organism is Gram-positive. A similar species inhabits the caecum of the horse (Baker & Martin, 1939).

Amylosarcina maxima (pro tem.). This conspicuous species is distinguished by its size (1·5–3 μ) and powerful iodine reaction, and by the exceptionally strong affinity which it shows for both methylene blue and the Romanowsky combinations. Durations of staining or concentrations of stain insufficient to colour any of the previous species bring the sarcinae clearly into prominence. As the genus indicates, packets of 4, 16, etc., units predominate, though less strictly co-ordinated aggregations occur. The organism is Gram-negative, thus differing from the genus *Sarcina*. With Romanowsky combinations the cell contents are stained a deep purple and are surrounded by a thick clearly defined reddish sheath whose outer mucilaginous surface forms a pink slime capsule in which entire groups of organisms are enclosed. The presence of this capsule can also be demonstrated in negative mounts. Both sheath and capsule are aniodophile. A similar species has been observed in the caecum of the red squirrel.

Amylosarcina minor (pro tem.). It is doubtful whether this species is distinct from the above, but it differs conspicuously from it, not only by its smaller size (0·5–1·2 μ), but also by the characteristics of its grouping, in which the union of the cells as pairs is always closer than the union of these pairs as tetrads. The matrix in which the units are embedded is firm and does not exhibit a slime capsule. The organism does not exhibit the intense affinity for methylene blue, etc., characterizing *A. maxima*.

Amylococcus. With the exception of *A. minor* all the types of micro-organisms instanced above show such clear-cut characteristics that they are readily distinguished. In addition, a group of micro-organisms included under the genus *Amylococcus* is always encountered, this group differing in a definitively streptococcal alignment. A number of variants will be indicated without allocation to each of specific status. These Amylococci are amongst the most widely distributed iodophile organisms of the gastro-intestinal tract and are found in the caecum and/or rumen of numerous herbivora, rodents, graminivora, etc. Since their staining reactions and minute structure have been described (Baker, 1933) only those morphological features which suffice for their recognition in iodine or film preparations are indicated.

Giant variants. Under this term are included cocci of 1·9–2·5 μ diam. forming short streptococcal chains of 2–8 (rarely 16) individuals. Typical schizomycete division is observed, the line of fission being devoid, initially, of iodophile substance. Two subvariants are present. In one, which may be described as *compact*, the daughter individuals remain closely united, thus forming short more or less rigid rectilinear filaments; in the other, which may be termed *loose*, the individuals are separated by wide intervals, connexion being maintained by extremely attenuated threads of viscid mucilage. The filaments of this variant are flexible and may become elaborately curved or twisted. In all cases a clearly defined aniodophile sheath is present whose outer surface is mucilaginous.

Medium variants. These display smaller dimensions of the individuals (1·2–1·5 × 15 μ) and greater length of the chains which must be assigned to the 'longus' type. The 'compact' association of components prevails in by far the larger number.

The micro-iodophile species

In addition to the large species described above and accumulating in fraction D, fraction E included, as members of the free iodophile population, immense numbers of small or micro-iodophile types. Similar micro-organisms were also found as members of the fixed population adhering to starch grains and plant structures. These forms fell under three chief groups, namely:

(1) Isolated iodophile cocci, diplococci and short or long chains.

(2) Curved vibrionic types.

(3) Short, slender non-sporing rods.

The activities of the first and second types in relation to the decomposition of starch and cellulose are considered below. Their morphology was not sufficiently distinctive to warrant the erection of new genera or species.

The aniodophile microflora

Together with the macro-iodophile species of fraction D and particularly with the micro-iodophile types of fraction E were found innumerable micro-organisms giving no colour reaction with iodine. These forms, which constitute the aniodophile microflora, included vast numbers of minute cocci and rods. In addition, the following types were always encountered and could readily be identified:

Spirochaetes. Included species were identical in morphology with those encountered in the human mouth and in the gastro-intestinal tract of other herbivora and graminivora such as the guinea-pig (Baker, 1933), horse (Baker & Martin, 1939), sheep and squirrel. These species of spirochaetes may be classified under the genera *Spirochaeta*, *Borellia*, *Treponema*, etc. (Bergey, 1939), but the value of such classifications is uncertain since in each host animal all transitions can be observed (Wenyon, 1926).

Fusobacteria. Closely associated with spirochaetes are types of the genus *Fusobacterium*. These micro-

organisms are long, slender, more or less curved, unbranched, non-motile, non-sporing and unsegmented rods, with sharply pointed ends. In the interior metachromatic granules can readily be demonstrated with polychrome blue or Romanowsky stains. Fuso-spirochaete symbioses are present in various situations, e.g. the human mouth, in Vincent's angina, and in certain wound infections. Similar associations to those encountered in the rumen have been observed in the caecum of guinea-pigs, horses, and squirrels.

Selenomonas. The organism observed in the rumen was identical with that present in the caecum of guinea-pigs and designated by Boskamp (1922) as *S. palpitans*, and was fully described by Baker (1933). The following points, which enable the micro-organisms to be identified readily either in fresh material or in stained preparations, may be noted. The individual cell is crescentic and 5–12 μ long and 0·8–1·8 μ broad. Situated medially beneath the cell membrane on the concave surface is a small compact spherical or oval body which stains strongly with nuclear stains and in Romanowsky films. Immediately above it is inserted a single long flame-like flagellum composed of fibrils cemented together by an interstitial substance. The medio-lateral insertion of the flagellum and its relation to the subjacent chromatin body serve to distinguish the species sharply from those of the genus *Spirillum* with polar monotrich or lophotrich flagellation. Equally characteristic in fresh preparations is the irregular spiral trajectory of the organism and its rapid spasmodic mode of progression (Boskamp, 1922).

Although the spirochaetes, fusobacteria and selenomonads collectively represent only a small fraction of the aniodophile population, counting procedures showed, in the course of *in vitro* incubations, that the numbers of these micro-organisms present may afford useful data in regard to the characteristics of the microbiological equilibria established under various experimental conditions.

Functional activities of the iodophile microflora

As stated, large numbers of macro- and micro-dophile species were closely adherent to the plant materials present in the rumen contents. By direct microscopical examination in polarized light it was possible to demonstrate that these members of the fixed iodophile population play a determinant role in the decomposition of starch and cellulose.

Action of iodophile micro-organisms in the disintegration of cellulosic structures

The role of iodophile micro-organisms in cellulose decomposition in the rumen of the ox is similar or identical to that already established in the case of other ruminant and non-ruminant herbivora. Direct microscopic observation shows an abundant iodophile microflora present upon the surfaces and within the interstices of the plant structures. The attack is manifested by the excision of clear-cut enzymatic cavities or lacunae in which the iodophile micro-organisms are enclosed. Within the lacunae the optical birefringence of the cellulosic substrate vanishes and its characteristic microchemical reactions disappear. Many of the structures subject to total disintegration embody components which are insoluble in 17·5% sodium hydrate, so that α-cellulose appears to be accessible to attack. Strongly lignified structures and cutin are unaffected. The iodophile types responsible for these changes include vibrionic forms and chains of macro- and micro-iodophile cocci. The disintegration of cellulose is already visible upon the surfaces of the large vegetable fragments comprised in fraction A. As disintegration proceeds the broken-down structures become detached, a process in which pectolytic bacteria, Protozoa, and the autonomous movements of the rumen variously participate. It may be emphasized here that although the mere occurrence of pectin and cellulose decomposition in the rumen may readily be demonstrated by purely biochemical methods, these procedures are insufficient to disclose the nature of the agencies concerned, still less definitely to allocate the changes taking place to any particular class of micro-organisms. Systematic application of the method of direct observation is essential to the final elucidation of the process of microbial decomposition and synthesis occurring in the rumen or cognate situations.

Action of iodophile micro-organisms in the disintegration of starch

In the rumen of the ox the iodophile microflora plays an important role in the disintegration of starch grains. The types primarily responsible are micro-iodophile amylococci whose components are of uniform diameter and form long chains. In the initial phase the micro-organisms are firmly attached to the surface of the grains upon which clearly outlined enzymatic cavities are excised. The attack proceeds from the periphery to the centre, and in the later phases the central residue is almost obliterated by the adherent micro-organisms. Finally, the grains are demolished, an interwoven knot of streptococcal chains marking their original locations. During the course of these changes, double refraction gradually ceases and the original blue-black reaction of the starch to iodine is replaced by a reddish reaction of diminished intensity. Similar features have been observed in the bacterial disintegration of starch occurring in the caecum of guinea-pigs (Baker, 1942*b*).

Although the volume of saliva passing into the paunch during rumination is large, its ptyalin content is small (Mangold, 1929). It seems probable, therefore, that in the ox the autonomous diastatic activity of the salivary excretion is, to a greater or less extent, reinforced by the bacterial disintegration of starch in the rumen.

Changes occurring in the rumen population during passage through the gastro-intestinal tract

Direct microscopic observation reveals marked changes in the microfauna and microflora of the rumen contents during their passage through the gastro-intestinal tract. Thus, it was possible repeatedly to confirm that the rumen Protozoa are absent from the lower regions of the large bowel. Further observation of faeces showed that in addition to the Protozoa, the majority of the iodophile micro-organisms had been destroyed. Where this destruction was not complete, as in a certain proportion of amylococci, the intermediate phases of disorganization could be observed. These included, together with a progressive loss of iodophile polysaccharide from the cell contents, a marked erosion and structural deformation of the substances of the sheath. At the same time, it could be observed that these bacterial and protozoan cytolyses had been accompanied by the establishment in the lower bowel of a new microbial population possessing characteristics distinct from that of the rumen. Attention will be confined to a single issue which has a direct bearing on the problems under consideration. In films made from faeces, in comparison with films from rumen contents, a marked increase of spore-bearing bacilli was always apparent. Amongst these were numerous slender Gram-negative rods with spherical or oval, terminal or subterminal spores. Micro-organisms of this kind are known to include, as well as proteolytic forms such as *B. putrificus coli*, the majority of forms to which have been attributed, since the period of Omelianski's researches, the major role in the anaerobic decomposition of cellulose in the gastro-intestinal tract (Khouvine, 1926; Pochon, 1935). Various species of such anaerobic cellulose-splitters may be readily obtained, at least in mixed culture, by the inoculation of mineral salt media, not only with the rumen or caecal contents, but also with faeces, material from dung-heaps and soil. It has been demonstrated by direct microscopic observation that these forms, although abundant in faeces, are not those primarily concerned with the breakdown of natural cellulosic substrates either in the rumen of ruminant or the caecum of non-ruminant erbivora (Baker, 1939). It would seem, therefore, that the claims advanced rest upon a misinterpretation of the results of pure-cultural investigation. A more probable interpretation would be that the spores of those micro-organisms are widely distributed throughout the alimentary canal and so readily germinate *in vitro* when inoculated into appropriate media; but that *in vivo* they discover suitable conditions for their development chiefly in the lower regions of the large bowel. To them, therefore, can be attributed only an ancillary role in the disintegration of cell-wall structures in the gastro-intestinal tract, their activities being confined to the decomposition of such unlignified residues as have escaped destruction by the micro-organisms of the rumen or caecum.

Factors concerned in the disorganization of the rumen population

(*a*) *Disorganization of the Protozoa*

The rumen Protozoa on entering the abomasum are annihilated in vast numbers: examination of abomasum material showed that the cytoplasm of such organisms was disorganized, but that the cuticular membranes of the larger Entodiniomorpha were unaffected. To obtain more accurate information regarding the changes taking place, enzyme preparations were made from (washed) formalized protozoan sediment (fraction B). By this means the occurrence of autolysis was eliminated. Permanent Heidenhain mounts of the digested material were secured permitting a more detailed study of the process than is possible with temporary preparations.

Pepsin. Examination of pepsin-hydrochloric acid preparations after 24 hr. at 40° C. showed that, in the larger species, the entire ectoplasmic region had been dissolved. The paraglycogen reserves had often become dissociated from their matrix, but did not appear to have undergone any further change, since individual granules accumulated in large numbers in the surrounding liquid. Similarly, the polygonal grains of the skeletal rods had become to a greater or less degree dislocated from their normal positions though retaining their original angular contours. In addition, a portion of the endoplasm surrounding the gastric sac had dissolved and all traces of the meganucleus had vanished, an observation confirmed in preparations stained with Heidenhain's haematoxylin, acetic iodine green, and picrocarmine respectively. In all the larger Entodiniomorpha the cuticula remained intact, but in the smaller species the regions surrounding the cytopharynx and cytopyge were dissolved. The cirri of the ciliophores and the cilia of the Isotrichidae present were destroyed. These observations, which confirm the results obtained by direct examination of gastric contents, demonstrate that the cell proteins of the Protozoa are readily accessible to peptic digestion. Control preparations in 0·2% hydrochloric acid were unaffected.

Trypsin. Essentially similar changes to those described above were observed in trypsin preparations containing 0·5% sodium carbonate, but here no paraglycogen granules could be observed in the centrifuged deposit, so that their actual solution may be inferred. As in pepsin preparations the polygonal granules of the skeletal structures, though dislocated from their matrices, did not appear to be otherwise affected. These results indicate that both the proteins and paraglycogen reserves are accessible to tryptic digestion. Controls in 0·5% sodium carbonate were unaffected.

Autolysis. Since in such non-ruminants as the horse the protozoan microfauna of the caecum may disappear during passage through the large bowel, it may be inferred that autolysis can also co-operate in the process of elimination.

(*b*) *Disorganization of the iodophile microflora*

The role of the Protozoa in the elimination of iodophile micro-organisms from the rumen contents has been described. The anatomical situation and relationships of the rumen evidently make it possible in principle for the activities of the Protozoa to be supplemented to a greater or less extent by the direct action of the gastric and pancreatic secretions. This point was investigated as follows:

Action of pepsin. To prevent the occurrence of autolysis, the investigations were made either upon boiled or formalized samples: in the latter case formalin was removed from the material (fraction D) by repeated washing in water and centrifuging. The activity of the enzyme preparation was checked by the use of Metts' tubes. A solution of 1% commercial pepsin in 0·2% hydrochloric acid was employed. The samples were incubated at 40° C. No action was observed on the species of the macro-iodophile population over a period of 5 days, which indicates that gastric secretions play little or no part in the digestion of iodophile micro-organisms.

Action of trypsin. The effects varied in degree according to the brand of trypsin employed. Gurr's preparation, dissolved in 0·5% sodium carbonate, showed an effect on boiled samples after a period of 24 hr. Controls with a 0·5% sodium carbonate were unaffected. The numerous colonies of *Amylobacterium asteroides* present in the initial sample were digested. *Amylospirillum* proved more resistant to attack. Partial digestion of *Amylosarcina* was established.

Relative rates of digestion of Protozoa and iodophile micro-organisms

From these observations it is clear that while Protozoa are accessible to gastric digestion, the iodophile microflora is almost unaffected. Similar comparative experiments demonstrated that even though some digestion of iodophile micro-organisms by trypsin could be detected, this occurred far more slowly than in the case of the Protozoa. The digestion of the iodophile microflora by the Protozoa results therefore in the conversion of a certain proportion of the products of microbial synthesis into the more readily digestible form of protozoan protein and carbohydrates.

Autolysis as a factor in the disorganization of the iodophile microflora

That the action of the Protozoa and the digestive enzymes of the host animal do not exhaust the factors operative in the observed elimination of the iodophile microflora, is indicated by the fact that this elimination is quite as conspicuous in non-ruminant as in ruminant herbivora (Baker & Martin, 1939). Since, in the former, the caecum replaces the rumen as the locus of microbial proliferation, it is clear, from the anatomical situation of the organ, that elimination cannot be due to the action of either the gastric or pancreatic secretions. Certain experimental results suggest that in these cases bacterial autolysis is the primary factor in elimination.

Autolysis in non-ruminants

Since the physiological conditions established in the caecum are less complex than those in the rumen, a preliminary experiment was made with the caecal contents of a guinea-pig. An account of the iodophile microflora of this animal was given by Baker & Martin (1937*b*). Material was collected post-mortem from a chloroformed animal. A sample was formalized and set aside as a control. The remainder was diluted with several volumes of normal saline saturated with chloroform. This suspension was divided into two portions, one of which was placed for 2 min. in a boiling water-bath. A few drops of chloroform were added to replace that lost by evaporation. Both fractions were placed in the incubator at 37° C. for 24 hr.

Examination of the formalized sample demonstrated the existence of an abundant iodophile microflora. Much residual starch was present which was in active process of bacterial decomposition, the surface of the granules being covered with a dense layer of iodophile micro-organisms. In the boiled fraction the starch grains had become swollen and distorted, though their iodine reaction and that of the entire iodophile microflora was substantially preserved. Examination of the unboiled fraction showed that, whilst the iodine reaction of the starch was unaffected, that of the iodophile microflora, including the micro-organisms attached to the surface of the granules, had disappeared.

From the differential behaviour in this respect of granules and bacteria, it may be concluded that the loss of reaction was not due to the enzymatic activity of the external medium but to changes taking place within the micro-organisms themselves.

Autolysis in ruminants

Exactly comparable results were obtained subsequently with rumen contents removed from a cow immediately after death and diluted with saline saturated with chloroform. Autolysis commenced with the disappearance of iodophile substance from the interior of the micro-organisms and afterwards extended to the substances of the sheath.

These facts indicate that under certain circumstances bacterial autolysis alone may account for the disappearance of the iodophile microflora from the faeces; indeed, this may normally be the case in non-ruminant herbivora. In ruminants, on the other hand, it is apparent that a more complex state of

affairs prevails and that autolysis may be supplemented, first, by the activities of the specific rumen Protozoa, and secondly by the action of the pancreatic secretion. Furthermore, it appears probable that the extent to which any one of these three potentially available routes of elimination is canalized, will devolve upon the simultaneous operation of several factors, including the actual balance of the diet components fed to the animal (Baker, 1942*b*).

Attention may be directed to the consideration that the number of iodophile bacteria eliminated by the Protozoa is dependent upon the density of the protozoan population. This is itself controlled by a variety of factors including the amount of available polysaccharide in the form of starch, the proportion of greenstuff in the diet, and the *p*H of the rumen contents (Westphal, 1934). The latter in turn depends upon the nature of the fermentative process, particularly upon the relative proportion of acids and bases in the liberated products of decomposition. When it is recalled that the decomposition of polysaccharides in the rumen is accompanied by the liberation of carbon dioxide and formation of organic acids, whereas that of urea (in urea rations) is attended by vigorous production of ammonia, the relation of these factors to the establishment of microbiological equilibria becomes apparent.

Discussion

This paper deals with two aspects of the microbiology of the rumen: (1) the systematic description of the species normally present, and (2) the elucidation of the functional activities of the micro-organisms described. The former continues the previous studies of the author in this field (Baker, 1933; Baker & Martin, 1937*a* and *b*, 1939); the latter constitutes part of an investigation undertaken in collaboration with the Hannah Dairy Research Institute (Owen *et al.* 1941). The end in view was to determine by direct microscopical observation the changes in microbial population which accompany the biochemical changes consequent upon the *in vitro* incubation of rumen contents with urea and certain other substances. The biochemical aspects of the work have been dealt with by Pearson & Smith (1943). They have assumed that if the micro-organisms during the period of incubation are the same as those present in the fresh rumen contents, then the biochemical processes demonstrable *in vitro* will afford insight into those occurring *in vivo* in the rumen of the fistula animal. Failure to secure this control vitiated the conclusions drawn by many investigators (Mangold, 1929; Krebs, 1937). For the assumption to be valid it is essential that the picture formed of the normal rumen population should be a representative one, and that this is so is demonstrated by the following facts:

(1) All the chief groups of micro-organisms present were represented both in post-mortem material collected at Guildford and in samples taken from the fistula animal at Kirkhill.

(2) The same groups were observed in all the fistula samples examined over the period 3 Mar. 1940–30 June 1942.

(3) During this period the animal, besides being put to grass, received a wide range of diets, including hay, together with blood meal, urea, and cereal rations.

It is clear, therefore, that the microbial population of the rumen is persistent and stable, and that its broad qualitative features are independent of the breed of the animal, the locality of the herd or, in the examples instanced, the nature of the diet.

By the use of counting cells it has been found possible to supplement the qualitative by a quantitative characterization of the changes occurring in the microbial population in *in vitro* incubations.

Functional significance of the rumen micro-organisms. Pearson & Smith's work (1943) is primarily concerned with the role of rumen micro-organisms in the synthesis of protein from non-protein nitrogen. A discussion of the microbiology of this process is not yet possible, since the requisite data, which include the results forthcoming from the systematic application of counting procedures are still in process of completion. In regard to the carbohydrate transformations occurring in the rumen, however, the facts presented permit definite conclusions which confirm and extend those advanced by the author in previous publications. Thus, the rôle of iodophile micro-organisms in the decomposition of starch and cellulose has been confirmed, an elimination of iodophile micro-organisms during passage through the alimentary canal is established, and the role of Protozoa in effecting this elimination becomes apparent. Moreover, it has been shown that, in consequence of this process, a conversion of less to more digestible microbial products is continuously effected. Finally, the conjectured operation of autolysis as an additional factor in elimination has been verified by observation on fresh material and by *in vitro* experiments. The author's present views on these subjects are epitomized in *Nature* (1942*c*).

In conclusion, it will be profitable to isolate for emphasis a single issue upon the exact appreciation of which the solution of many problems in rumen microbiology pivots. Direct microscopical observation establishes that iodophile micro-organisms play a major role in the disintegration of *cellulose* in the rumen of ruminant and in the caecum of non-ruminant herbivora. It is clear, therefore, that the microbial decomposition of cellulosic substrates is throughout accompanied by microbial synthesis of an iodophile polysaccharide. No less evident is the fact, to which attention has been drawn by biochemical investigators, that in the course of this process organic acids are liberated. Two main routes, therefore, are in principle available for the effective transference of polysaccharide from the cellulosic

materials ingested to the tissues of the animal: we may surmise a direct absorption and subsequent recondensation of the initially liberated products of decomposition, or we may assume that assimilation is indirect, and that the animal is able productively to utilize the products of microbial synthesis. In view of the fact that elimination of iodophile micro-organisms during or previous to passage through the large bowel has been demonstrated, this second hypothesis can now lay claim to serious consideration. Moreover, the two hypotheses are not necessarily incompatible, since the simultaneous canalization of several routes of transference is not in principle excluded. At this juncture the problem assumes a quantitative form, since the respective value of the products of microbial decomposition and synthesis devolve both on the relative amounts of each produced, the facility with which they can be absorbed, and their relation to the metabolic requirements of the animal. It is evident, therefore, that while this central problem of rumen microbiology can now be stated with precision, its solution must await the outcome of results forthcoming from related fields of investigation.

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The comparison of sheep-tick populations (*Ixodes ricinus* L.)

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Experiments in sheep-tick control require the comparison of tick counts on sheep pastured on an infested grazing or on a blanket dragged over the grazing. The former should include only attached females on axillae, forearms, head, neck and chest.

The distribution of tick counts on a group of sheep is in general not fitted by the normal distribution, nor, although it is positively skewed, by the Poisson. There is evidence that the distribution could be derived from a population distributed according to a negative binomial. Unless the data are more than ordinarily skewed (in which case, a 'normalizing' square-root transformation may be necessary), the best available method for comparing mean tick counts on sheep is the direct t-test using actual tick numbers. Within the range 0·3–103·7 ticks per sheep, the regression of standard deviations on means is linear and was calculated as $s = 0{\cdot}477\bar{x} + 1{\cdot}246$.

For estimating the significance of small differences between dips or ground population densities, tick counts on groups of five sheep are inadequate. Groups of twenty or more sheep should be employed according to the magnitude of the difference required to be proved significant. For showing the trend, only, of tick activity throughout a season on a particular section of land weekly counts on ten sheep are adequate provided the same ten sheep are always used. The sources of the large variation in the tick counts of individual sheep are pointed out, with suggestions as to how this variation may be reduced.

In blanket dragging, counts of nymphs are best for estimating population densities. Drags may be limited to 25 yd. A differential equation is given whereby the nymphs lost in the course of a drag are taken into account. This does not surmount the difficulty that uniformity of vegetation surface influences the efficiency of the blanket, which should therefore be used for comparisons only when the vegetation surfaces are of similar uniformity. A worn blanket picks up fewer ticks than a less worn blanket. The distribution of nymphal blanket counts is similar to that of female tick counts on sheep in that it is not in agreement with the Poisson law although positively skewed. It is in closer, though far from satisfactory, agreement with a 'contagious' distribution.

Because of the effect of changing meteorological conditions on tick activity, the densities of tick population (tick activities) on different plots must be compared by dragging the plots simultaneously. A virgin stretch of ground is necessary for each drag in each season. In such comparisons, the precision can more easily be increased by increasing the number of 'occasions' (days) of simultaneous draggings than by increasing the number of drags per 'occasion'. Two, or three, drags per plot are sufficient provided the number of 'occasions' is not less than 20. The significance of plot differences in density may be calculated from the analysis of variance of drags.

For plots less than 3 acres, the blanket method is easier; for large areas, especially with varied vegetation cover, tick counts on sheep are preferable.

Introduction

Since MacLeod & Gordon (1932) proved the sheep tick, *Ixodes ricinus* L., to be a disease vector the problem of its control has received more attention. The pest is a three-host tick with a life cycle varying from about 1½ to 4½ years, approximately 3 weeks of which are spent on its hosts and the remainder of the time on the ground (MacLeod, 1932, 1939*b*). Various control methods are under test including (1) dipping of sheep—the chief domestic host, and (2) the improvement of hill grazings—poor grazings apparently providing the optimum conditions for survival of the sheep tick.

Estimates of control effects may be made in two ways: by counting the ticks (*a*) on sheep pastured on an infested grazing, or (*b*) on a blanket dragged over the grazing. Tick counts on sheep show the immediate effects of a dip by the proportion of ticks killed and the duration of the killing property. Tick counts both on sheep and on the blanket are used to estimate tick population-reduction effects on an infested grazing, whether by hill-land improvement or by serial dipping of sheep, and to compare ground densities for ecological purposes.

Tick counts on sheep

(i) *Method of counting*

Because of drawbacks connected with the counting of larvae, nymphs and males, MacLeod (1939*a*) counted only attached females on the axillae, forearms, head, neck and chest of individual sheep. Moore (1938, 1939) included female ticks on other parts of the body, while Linton (1941) counted attached nymphs as well as females, body areas unspecified. MacLeod's method is adequate, and confusion in comparisons would be avoided if it were adopted.

(ii) *The distribution of tick counts on sheep, and method of comparing mean tick counts*

The distribution of tick counts on sheep was studied to find a statistical method of comparison. Table 1 gives the frequency distributions of ticks in ten groups of sheep in ascending order of means. In each group the individual sheep were of the same age class, all 'running together' on the same grazing and all counted at one gathering.

Fisher (1941 *a*) examined the tick counts in Table 1 and, using cases 2 and 4, showed that the distribution could be derived by random sampling from a population distributed according to the negative binomial of the form $(q-p)^{-k}$, where $q=1-p$, and k is positive. He states: 'With the negative binomial we ordinarily require to estimate the exponent in addition to the mean of the distribution. This can be done from the first two moments, but the process has been recognized as inefficient and in the present note the theoretical efficiency is calculated so as to make it easy to judge in practical cases whether a more exact fitting by maximal likelihood is required.' In a private communication (in answer to the query whether the negative binomial could be used in making tests of the significance of the difference between mean tick counts on groups of twenty sheep, and, if not, whether some 'normalizing' transformation of tick numbers was permissible) Fisher adds: 'I think the negative binomial is likely to be increasingly used in these and analogous

TABLE 1. *Frequency distributions of ticks in ten groups of sheep*

$\bar{x}$=mean; x=no. of ticks; n=total no. of sheep; f=no. of sheep and $f=0$ is omitted in all cases; v=variance.

Case 1. $\bar{x}=0{\cdot}84$, $n=83$, $v=2{\cdot}517$

x	0	1	2	3	4	5	6	11
f	46	24	7	2	1	1	1	1

Case 2. $\bar{x}=3{\cdot}25$, $n=60$, $v=5{\cdot}821$

x	0	1	2	3	4	5	6	7	9	10
f	7	9	8	13	8	5	4	3	1	2

Case 3. $\bar{x}=4{\cdot}23$, $n=30$, $v=10{\cdot}64$

x	0	1	2	3	4	5	6	7	9	11	15
f	1	5	3	6	6	1	3	1	2	1	1

Case 4. $\bar{x}=6{\cdot}56$, $n=82$, $v=34{\cdot}34$

x	0	1	2	3	4	5	6	7	8	9	10	11	13	14	15	16	19	21	22	23	25
f	4	5	11	10	9	11	3	5	3	2	2	5	2	2	1	1	1	1	1	1	2

Case 5. $\bar{x}=11{\cdot}05$, $n=40$, $v=49{\cdot}00$

x	0	1	2	3	4	5	6	8	9	11	12	13	14	15	16	17	18	21	28	29
f	1	1	3	1	3	1	2	4	4	1	2	4	2	1	1	3	1	3	1	1

Case 6. $\bar{x}=22{\cdot}17$, $n=36$, $v=96{\cdot}51$

x	5	9	10	11	12	15	16	18	19	20	21	23	24	25	26	27	30	31	34	36	42	54
f	1	1	3	1	2	2	1	1	1	2	3	1	3	3	2	2	2	1	1	1	1	1

Case 7. $\bar{x}=25{\cdot}41$, $n=86$, $v=282{\cdot}6$

x	2	3	4	5	6	7	8	9	10	11	14	15	16	17	18	19	20	21	22	23	24	25	26	28
f	1	1	2	3	3	4	4	4	1	4	2	1	2	1	1	2	1	2	3	1	1	4	2	1
	30	31	32	33	34	36	37	38	39	40	41	42	44	47	48	49	50	51	53	58	62	67	70	78
	2	1	3	4	3	1	1	3	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Case 8. $\bar{x}=33{\cdot}00$, $n=33$, $v=219{\cdot}4$

x	6	12	13	17	18	20	23	24	28	29	30	31	32	34	36	37	39	43	44	47	49	53	60	63
f	1	1	2	2	1	2	1	1	1	1	1	3	1	1	1	2	2	1	1	1	1	2	2	1

Case 9. $\bar{x}=40{\cdot}58$, $n=24$, $v=307{\cdot}9$

x	17	18	21	23	25	27	29	31	35	36	44	45	47	48	50	52	53	65	67	92
f	1	1	2	1	1	1	1	1	2	1	1	2	1	2	1	1	1	1	1	1

Case 10. $\bar{x}=85{\cdot}55$, $n=20$, $v=2292{\cdot}0$

x	22	26	27	31	47	62	64	66	74	78	84	87	90	100	116	161	162	168	180
f	1	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1

Cases 1–5 and 7 were examined for agreement with the Poisson distribution, which would be followed if ticks were picked up at random and independently of one another. Cases 2 and 3 yielded $\chi^2_{[4]}=8{\cdot}319$ and $\chi^2_{[2]}=2{\cdot}346$ respectively; case 1 gave $\chi^2_{[2]}=7{\cdot}280$; the three remaining cases gave very high χ^2, beyond the 5 % level. Although there is a hint that female ticks may be randomly distributed on sheep when the mean is low, it is clear that one or more factors interfere strongly with the randomness of the distribution in general (cf. variances in Table 1 which range from about 2 to 27 times the mean).

studies.... In respect to the validity of comparisons based on means, other things being equal, the mean is certainly an efficient statistic for this distribution, though, if the lots compared differ also in the parameter k it clearly does not, by itself, give the complete comparison. Pending further knowledge, there is certainly nothing to prevent one supplementing comparisons based on means with additional comparisons using, e.g. square root, or logarithm, of the tick number. If it were found that these often showed significant differences where the arithmetic mean did not, this would be sufficient evidence in favour of their use, without further mathematical investigations, which seem at present rather obscure'.

The question arises therefore whether a simple transformation of the tick counts (in Table 1) such as their logarithms or square roots gives a distribution more nearly normal. The logarithm of the tick number ($x+1$ when $x=0$ occurs in the sample—Williams, 1937) gives in general an opposite but slightly less skewness to the curve of the distribution. Using the square root of the tick number an intermediate curve is obtained in which the frequency, and the range, on either side of the mean tend to equalize, while the general resemblance to the normal curve is much closer than with the skewed tick numbers and log tick numbers.

Since the mathematical equipment is not yet forthcoming for making tests of significance with the negative binomial distribution, and since from the data available so far there is no guarantee that it is the true distribution, the results of t-testing with actual tick numbers as opposed to square roots of tick numbers were compared. The data of thirty-two experiments were examined in each of which tick counts on two groups of twenty sheep had been made to compare two dips, the infestations of fat and lean sheep, or the ground density of tick population on two areas. In only two cases out of the thirty-two would a different conclusion (as to significance of difference between means) have been reached at a 5% significance level, and in these two cases the non-significant values are on the borderline:

t (actual numbers)	t (square roots)	Degrees of freedom
1·977	1·662	38
1·951	2·068	38

In the remaining thirty cases the conclusion with regard to significance was the same by both tests. It was decided therefore not to transform to square roots unless the data be skewed more than usual. With the latter proviso the best method available for comparing mean tick counts on sheep is the direct t-test (Fisher, 1941 *c*) using the actual tick numbers. When the means of more than two groups of sheep are to be compared the analysis of variance will be employed.

(iii) *Variation in tick counts on small groups of sheep as affecting their comparison*

Statistical considerations are important in the branches of tick research which depend on the comparison of average tick counts on small groups of sheep. In the course of this work, it was noted that the scatter of tick counts on a small group of sheep running together on the same ground is often considerable; the range is frequently $\times 2$–3 the mean. For example, the totals of ticks on each of ten sheep drawn at random from a flock of about 100 running on one relatively small section of hill in May 1941 were: 12, 20, 52, 69, 74, 81, 94, 103, 109, 243. It is consequently unsafe to base conclusions on the simple difference between mean tick counts on small groups of sheep chosen at random from a flock, without examining this difference in relation to its standard error. This is illustrated by data from a dipping experiment showing the tick counts for two groups of ten sheep randomly chosen from the two dipped flocks which were running together:

Dip A:
31, 39, 8, 34, 58, 25, 22, 48, 6, 21 Mean 29·2
Dip B:
60, 19, 68, 54, 46, 2, 72, 104, 151, 20 Mean 59·6

The difference between the means, 30·4, seems to show an overwhelming superiority for dip A. But by actual tick numbers $t=2{\cdot}053$ and by square roots $t=1{\cdot}762$, both values, with 18 degrees of freedom, being below the 5% level, though the first is very close to it. The large difference between the means is not significant, since a difference of this order could have occurred by chance more frequently than once in twenty draws of pairs of ten sheep from a flock in which all the individuals were treated with the same dip. This finding is borne out by other experiments with the dips; sheep treated with dip A or dip B reattain the normal level of infestation, as judged by undipped controls, at about 3 weeks after dipping; and the counts given above were made on the 20th day after dipping.

From 115 cases (tick counts on groups of 10–86 sheep each) with means ranging from 0·3 to 103·7 ticks per sheep, the relation between mean and standard deviation is linear within this range of means. The regression of standard deviations on means was calculated as

$$s=0{\cdot}477\bar{x}+1{\cdot}246,$$

where s = standard deviation, and $\bar{x}$ = mean.

Where two groups of sheep are of equal size, the 0·05 probability that the observed difference between their means occurred by chance (i.e. could occur about 1 in 20 times by random sampling from the *same* population) is reached when that difference is equal to $\times 1{\cdot}96$ the standard error of the difference between the means. When the samples (groups) are from the same population, twice (i.e. 1·96) the standard error of the difference between the means

is approximately equal to $\times 3$ the standard error of the group mean. Employing this approximation in conjunction with the expression relating standard deviations and means (see above), the difference between means required for the 0·05 probability can be roughly calculated for any particular mean. Table 2 was constructed for selected means and shows, for example, that if one group of twenty sheep has a mean of 15 ticks per sheep, another group of twenty must have a mean about 5·64 ticks greater or less than 15 before the difference between the pair can be regarded as significant; with groups of ten and five sheep, the necessary difference would be about 7·95 and 11·28 ticks respectively. Table 2 is only a rough guide, but it is useful for indicating when a *t*-test is unnecessary, i.e. when the difference is clearly not significant. (Values for means not given in Table 2 can be obtained by graphing the data in the table.)

TABLE 2. *The order of differences between arithmetic means required for significance (i.e. $P=0{\cdot}05$)*

Means in the region of	Difference between arithmetic means for $P=0{\cdot}05$ for groups of		
	20 sheep	10 sheep	5 sheep
1 tick	1·16 ticks	1·63 ticks	2·31 ticks
2 ticks	1·48 ,,	2·08 ,,	2·95 ,,
3 ,,	1·80 ,,	2·54 ,,	3·60 ,,
5 ,,	2·44 ,,	3·45 ,,	4·86 ,,
10 ,,	4·05 ,,	5·70 ,,	8·07 ,,
15 ,,	5·64 ,,	7·95 ,,	11·28 ,,
20 ,,	7·26 ,,	10·26 ,,	14·52 ,,
30 ,,	10·47 ,,	14·70 ,,	20·82 ,,
50 ,,	16·89 ,,	23·85 ,,	33·69 ,,
100 ,,	32·91 ,,	46·44 ,,	65·61 ,,

Past work (Lyle Stewart, 1936*a*, *b*, 1939*a*, *b*, 1940; MacLeod, 1934, 1939*a*; Moore, 1938, 1939), especially on dips, rested on means of groups of sheep as small as five, very seldom more than ten and twelve, the upper limit. The above finding (see Table 2) shows the need for care in accepting conclusions based on the simple difference between arithmetic means alone especially where means only are given. It also indicates that groups of five sheep are inadequate for estimating the significance of smaller differences in the comparison of the effectiveness of dips (dip technology) or the comparison of tick activities and, hence, tick population densities (ecology). Groups of twenty or more sheep should be employed, the number exceeding twenty according to the magnitude of the difference required to be proved significant. Where more than three or four groups of sheep must be examined on one day, a group size greater than twenty is not practicable even for two tick-counters. If groups larger than twenty, e.g. thirty, are necessary, there is no objection to counting ten from each group on each of three successive days so that comparisons between the totals of thirty would be unbiased by day-to-day differences.

For showing the trend of tick activity throughout a season on a particular section of hill land, weekly counts on ten sheep are adequate provided the same ten sheep are always used.

(iv) *Sources of variation in tick counts on sheep and their elimination*

MacLeod (1939*a*) showed that sheep of different age classes have different susceptibilities to tick infestation when he demonstrated that hoggs (1-year-old sheep) had on average a lower infestation than ewes (2–5 years old). But even in the same age class there is considerable variation in infestation levels as the counts already given show. This variation arises from a variety of sources, including:

(*a*) The patchy distribution of ticks on the ground: Milne (1940) and Cameron (1941) (unpublished reports) showed that tick population density varies with vegetation type, which, in turn, varies largely over comparatively small areas of a hill grazing. Since sheep are 'hefted' in groups of 100 or more to well-defined sections (hefts) of the hill, and since individuals tend to keep to their 'own' part (cut) of the heft, different sheep must be encountering different levels of tick population density on the ground over which they graze.

(*b*) The individual susceptibility of sheep of the same age class to infestation: Milne (1940, 1941; unpublished reports) also showed that sheep in good condition carry about half as many ticks as sheep in poor condition on the same ground at the same time.

(*c*) The individual activity of sheep in search of food: the more ground a sheep covers the more ticks it may pick up.

In choosing sheep for test groups, nothing can be done regarding (*c*). For (*a*) the sheep chosen should all come from one of the natural divisions of the hill, that most uniform in vegetation type being selected. For (*b*) care should be taken that the average condition of one group of sheep is not obviously higher than that of the other: a few fat sheep in one group may make a big difference to the mean tick count when the other group is uniformly in medium or poor condition. Where there is doubt, experiments need repeating with reversal of groups. Having selected a reasonably uniform batch of sheep, the assignment of individuals to experimental treatments must be random if unbiased results are to be obtained.

TICK COUNTS ON THE BLANKET

(i) *Method*

When active, the tick climbs to the plant tips and clutches any object which passes within reach. Advantage is taken of this in blanketing. A 'single' blanket attached to a cross-bar at the front end is dragged over the ground by a long cord (MacLeod, 1932). Larvae, nymphs, males and females may all be found clinging to or crawling upon the woolly

hairs of the underside of the blanket. The unit drag is 50 yd. in length. At the end of each drag the various stages of ticks are counted and removed.

(ii) *The efficiency of the blanket in picking up ticks*

Early in the present work, it was frequently noted that a male or female present on the blanket at the beginning of a drag was absent at the end. (This observation was tested by the method below used for nymphs.) Males and females are either scraped off, or seize passing vegetation, or both. This, together with the relative scarcity of males and females on the blanket (cf. Table 5 and p. 247) even when upwards of 100 are found on sheep working the same ground, led to making nymph counts the standard of population density by the blanket method.

TABLE 3. *Analysis of thirty-six blanket drags showing number of nymphs present in the first and second halves of the drag length* (50 *yd.*)

				A. 5 drags						
25 yd.	0	0	0	0	0					
50 yd.	0	0	0	0	0					
				B. 5 drags						
25 yd.	0	0	0	0	0					
50 yd.	1	1	1	2	4					
				C. 10 drags						
25 yd.	1	2*	2*	1	1	2	2	4	2	5
50 yd.	2	3	3	3	3	4	4	7	6	15
				D. 10 drags						
25 yd.	1	2*	3	3	3	3	4	4	5	6
50 yd.	1	2	3	3	3	3	4	4	5	6
				E. 6 drags						
25 yd.	1	1	1	4	4	6				
50 yd.	0	0	0	3	3	4				
				Totals						
25 yd.	73									
50 yd.	103									

* See text.

Nymphs, although less affected probably because of their smaller size, are also subject to 'scraping off' or 'clinging off' as the following example shows. The experiment comprised thirty-six drags, a virgin surface for each drag, on an apparently uniform stretch of rough bent grass. For each drag, a count was made at 25 yd., the ticks being left *in situ* on the blanket, and a second count made at the completion of the 50 yd., after which all ticks were removed. Table 3 shows that in six drags (case E) at least 1–2 of the nymphs present in the first half of the drag were lost during the second half. In C and D the same may have happened, for in the drags marked with an asterisk one nymph (specially noted for its position) was not present in the final total. Probably results would have been more striking if the blanketing had been done at the height of nymphal activity, i.e. 20–50 nymphs/50 yd. drag.

The 'scraping off' difficulty would be largely avoided for males and females as well as nymphs if the 'flagging' method of American workers (Philip, 1937) were used—provided the flag were examined after each sweep. This method consists in walking a fixed length, sweeping the vegetation scythe-like at each pace with a square of blanket tacked to a pole. It is laborious, and since the pace, the sweep length, and the weight of application of the flag cannot be controlled accurately when walking over rough ground, it is not to be preferred to the blanket method (when the number of drags is small) whereby an accurate area is sampled each time at a constant pressure.

From the data in Table 3 it may be calculated that the mean difference between individual pairs of 50 and 25 yd. drags, 0·83 nymph, is significant ($s=2{\cdot}079$, $t=7{\cdot}612$, D.F. $=35$; Fisher, 1941*b*). More nymphs are found on the blanket with 50 yd. drags than with 25, but it is likely that the latter shows more accurately the activity density on the ground dragged since the shorter the drag distance the less the 'scraping off'. The present work, however, is based on 50 yd. drags.

Prof. R. A. Fisher suggested a method whereby the ticks lost from the blanket in the course of a drag may be taken into account: 'Suppose that, in dragging the blanket, there is an accretion of a ticks per yard on the average, and a loss proportional to the number of ticks carried at the moment. On this basis one has the differential equation

$$\frac{dy}{dx}=a-ky,$$

where x is the distance traversed, y the number of ticks at any stage, a the rate of accretion measuring the tick population of the pasture, and k a factor representing the rate of loss, and probably dependent on the roughness of the pasture and other conditions. Putting in the condition that y is 0 when x is 0, the solution of this differential equation is

$$1-\frac{k}{a}y=e^{-kx}$$

so that if one has values $\frac{73}{36}$ and $\frac{103}{36}$ (means for counts at 25 and 50 yard points respectively, see Table 3) for two values of x, of which the second is double the first, one has the equation

$$\left(1-\frac{k}{a}\frac{73}{36}\right)^2=1-\frac{k}{a}\frac{103}{36}$$

or simply

$$\frac{k}{a}=\frac{43\times 36}{73^2}.$$

Using now the fact that the average number $\frac{73}{36}$ was attained after 25 yards drag, one has

$$e^{-25k}=1-\tfrac{43}{73} \quad \text{or} \quad k=\tfrac{1}{25}\log_e\tfrac{73}{30},$$

whence a, which measures the number of ticks per yard of pasture which get into the blanket, is

$$\frac{73^2}{43\times 36\times 25}\log_e\frac{73}{30}.$$

This comes to 0·12245 ticks per yard, or 3·061 per 25 yards, or 6·1225 per 50 yards, and so on.' In the case shown in Table 3, the mean number of ticks per 100 sq. yd. (blanket 2 yd. wide) which are picked up by the blanket, irrespective of whether they are lost or not, is 6·1225 nymphs. This would be an estimate of absolute activity per 100 sq. yd. only if the surface of the vegetation was such that the blanket touched the uppermost portion of every blade or frond in the area passed over so that every active tick could seize the blanket. The above correction does not take into account the difficulty presented when the blanket 'rides', but it is clear that counts from drags of different lengths cannot be pooled for a mean unless the correction is applied.

TABLE 4. *Comparison of bracken bed (plot 1) and bent grass (plot 2)*

	Case A Plots 1–2		Case B Plots 1–2
	3		3
18 Apr.	+15	30 May	− 6
	+19		− 4
	+ 5		− 7
	+21		+10
	+ 7		+ 1
	+16		− 6
	+11		−14
	+ 5		− 1
	+ 1		− 7
	+ 6		0
	+ 5		+ 1
	+ 9		+ 2
	+ 2		+ 1
	+27		− 4
16 May	+16		− 1
			0
		23 June	+ 1
	+156		−34
Means	+10·40		−2·00
Standard errors	± 1·958		±1·311

Uniformity of vegetation surface influences the efficiency of the blanket. In 1941 two plots were compared, plot 1, a bracken bed in which bents and other grasses were present, and plot 2, a fairly uniform patch of rough benty grass. Three drags per day were taken simultaneously on each plot on 32 days from 18 Apr. to 23 June. In Table 4 the data are divided at the date (30 May) when the bracken was 6 in. high on the bracken bed (plot 1). Only the mean difference in nymphal ticks per drag per day is given, and the method of testing the significance of differences is anticipated from § (iv) below.

Using the test for the significance of the mean of a unique sample on the two sets of data in Table 4 (Fisher, 1941*b*) in the case (A) $t=5{\cdot}496$, D.F. = 14, and in the case (B) $t=1{\cdot}572$, D.F. = 16. Thus when bracken was not interfering with the close application of the blanket to the general surface of the vegetational cover as a whole, tick population density on the bracken bed (plot 1), as measured by relative activity, was significantly higher than that on the rough grass (plot 2); when bracken was interfering, there was no significant difference between the two. Although ticks tend to reach the highest point in the vegetation, only a proportion of them will be living at or near the base of a bracken frond, and they do not, so far as is known, travel far horizontally for any purpose (MacLeod, 1938; Cameron,. 1939). Before the bracken grew the proportion of surface touched by the blanket on both plots was roughly similar. From the time the bracken was 6 in. high the blanket on plot 1 rode more on the tips of the fronds, thus touching much less of the general surface, and hence, very probably, missing ticks.

Totalling all the data between 18 Apr. and 23 June, $t=2{\cdot}841$, D.F. = 31. Thus it would not have mattered greatly if the growing bracken effect had not been noted, as the mean difference between the plots would still have been significant though not so large; but if sampling had been confined to the period 30 May to 23 June, it would have involved regarding population densities on the two plots as equal—so far as the data could show. In comparing two plots, therefore, uniformity of surface must be taken into consideration.

When a blanket is new its surface is covered with woolly fluff which gradually wears thinner and probably shorter in the course of dragging. A blanket (*a*) used for two seasons was compared with one (*b*) used for half a season. On a uniform stretch of rough grass, thirty-eight pairs of drags, i.e. seventy-six consecutive drags using the blankets alternately, showed a mean difference $(b-a)$ of $+1{\cdot}526$ nymphs per drag with $t=6{\cdot}08$, $P=<0{\cdot}01$. Thus the lesser-used blanket (*b*) picked up a significantly higher number of ticks, and this must be noted when more than one blanket is used in making comparisons.

(iii) *The distribution of blanket tick counts*

Blanket dragging is time-consuming and, since tick activity changes with meteorological conditions, the counts in Table 5 were made when activity was low (towards end of activity season) about midday when meteorological conditions (wind force, temperature, humidity, sun height and intensity, cloud, etc.) are relatively stable, and with assistance so that a sufficient number of drags could be done while conditions were stable. In Table 5 the theoretical frequencies in a Poisson series having the same mean as the observed distribution are given ('contagious' distribution, see p. 249). The distribution for nymphs on the blanket takes a form similar to that for females on sheep, i.e. positively skewed and in general not in agreement with the Poisson series. The same difficulties therefore arise in the treatment of blanket distributions as in sheep distributions.

TABLE 5. *Nymphal tick counts on the blanket*

	Nymphs per drag	No. of blanket drags: Observed	Poisson	'Contagious'
Case 1	0	18	17·60	18·20
	1	9	9·39	8·53
	2	2	2·50	2·55
	3	1	0·45	0·59
	>3	0	0·06	0·13
		30	30·00	30·00
	Means	0·53	0·53	0·53
Case 2	0	12	9·99	9·59
	1	5	10·99	10·95
	2	11	6·04	6·05
	3	2	2·20 (>2)	3·41
	>3	0	0·78	
		30	30·00	30·00
	Means	1·10	1·10	1·10
		$\chi^2_{[2]}$	8·06 $\chi^2_{[1]}$	8·47
*Case 3	0	10	4·74	8·39
	1	7	9·61	8·41
	2	6	9·74	7·23
	3	4	6·59	5·08
	4	5	3·34	3·19
	5	2	1·35 (>4)	3·70
	6	2	0·46	
	>6	0	0·17	
		36	36·00	36·00
	Means	2·03	2·03	2·03
		$\chi^2_{[3]}$	11·54 $\chi^2_{[2]}$	1·63
Case 4	0	9	2·37	10·02
	1	4	6·70	5·45
	2	4	9·46	5·97
	3	11	8·91	5·17
	4	6	6·29	4·06
	5	2	3·55 (>4)	9·33
	6	2	1·67	
	7	1	0·67	
	>7	†1	0·38	
		40	40·00	40·00
	Means	2·83	2·83	2·83
		$\chi^2_{[4]}$	23·00 $\chi^2_{[3]}$	9·83

* The drags were 25 yd. in length; in all others they were 50 yd.

† One drag yielding 15 nymphs. The following *totals* of males and females were found in the course of the drags:

Case	Males	Females
1	4	2
2	0	0
3	1	2
4	7	2

and never more than one female or male in a single drag.

(iv) *Method of blanketing for comparing two or more tick-infested areas, and method of comparing mean tick counts*

Tick activity as shown by counts on sheep for females and blanket counts for nymphs is continuously changing in two ways: (*a*) there is continuous change in level of activity during the active season due to fluctuating meteorological conditions; (*b*) superimposed on these continuous fluctuations, and correlated with the broader changes in meteorological conditions, e.g. average weekly temperature, is the seasonal rise from zero activity about mid-March to the peak during late April and early May followed by the fall to zero again about the end of June (Milne, unpublished work; cf. also MacLeod, 1939*a*). To blanket two areas, not simultaneously, but haphazardly, and independently on different days and at various times of day throughout the active season, total all the drags for each of the areas and compare the means is useless because, in effect, the distribution of counts for each area will be comprised of samples from many different populations. For comparative purposes it is necessary to make pairs of simultaneous observations as in Table 6.

TABLE 6. *Number of nymphs per blanket drag*

Three drags per day on each of two bracken beds (A and B) on 33 days from 18 Apr. to 25 June. Each pair of three drags was done simultaneously.

	A			B			Mean difference per drag (rounded figures) B−A
18 Apr.	4	7	4	11	43	34	+24
21	7	7	8	39	30	17	+21
22	5	5	2	11	18	24	+14
23	8	6	2	11	23	21	+13
28	6	6	4	19	21	20	+15
29	7	5	10	19	25	24	+15
30	3	8	3	13	25	23	+16
1 May	1	13	9	37	17	19	+17
5	5	6	5	19	21	19	+14
6	3	10	3	15	12	12	+ 8
8	11	6	9	27	17	28	+15
9	4	6	12	29	33	23	+21
19	2	0	1	17	25	7	+15
20	5	7	6	13	11	6	+ 4
21	3	1	1	11	9	5	+ 7
27	5	0	3	14	43	11	+20
28	6	0	3	16	17	28	+17
29	1	4	0	17	14	6	+11
30	2	3	0	10	10	6	+ 7
2 June	2	2	11	5	0	3	− 2
5	0	1	2	23	17	10	+16
6	5	2	2	10	8	18	+ 9
9	1	4	7	8	7	7	+ 3
10	3	2	1	2	2	11	+ 3
11	1	0	2	0	2	5	+ 1
12	3	0	4	2	3	5	+ 1
13	0	0	0	3	2	4	+ 3
16	1	3	2	4	4	7	+ 3
17	2	0	1	1	6	5	+ 3
19	2	1	1	5	3	4	+ 3
20	0	0	0	0	3	2	+ 2
23	0	1	0	1	1	3	+ 1
25	0	0	0	1	0	0	+ 0
	342			1302			+320

The data in Table 6, typical of blanketing results, are from two bracken beds, A and B, relatively uniform within themselves as to vegetation cover.

The figures in columns A and B are the totals of nymphs from each of three 50 yd. drags in each case. Pairs of three drags on each bracken bed were done simultaneously around midday on thirty-three occasions (days) from 18 Apr. to 25 June. Proceeding from the same side of each bed, a virgin strip of ground was taken for every drag. The last column in the table gives the mean difference per drag in rounded figures for ease in computation.

From columns A and B, the analysis of variance of the 198 drags may be calculated as follows:

	D.F.	Sum of squares	Mean square
(1) Occasions (days)	32	5964	—
(2) Plots	1	4654	4654
(3) Occasions × plots	32	2650	83
(4) Sampling error	132	3058	23
(5) Total	197	16326	—

To test the significance of the difference between plots, the mean square for 'plots' must be compared with the mean square for the interaction of 'occasions × plots'. In this case, $\frac{4654}{83} = 56{\cdot}07$, and the difference between plots A and B is significant. When only two plots are to be compared, the analysis of variance is unnecessary since a *t*-test (Fisher, 1941*b*) on the mean difference per drag per occasion is equivalent.

If E is the sampling variation per blanket drag and A an additional component of variation per blanket drag due to variation of plot differences between occasions, then the occasions × plots interaction will be: $V = nA + E$, if n drags per plot per occasion are used. Hence since $E = 23$, and $3A + E = 83$, then $A = 20$ in the above case. The variance per blanket drag to be used for testing plot differences is V; the variance of the mean of n drags is V/n and hence the variance of the difference of two means is equal to

$$\frac{2V}{n} = 2A + \frac{2E}{n} = s^2.$$

Substituting $A = 20$ and $E = 23$, s^2, i.e. the variance per occasion of the mean difference per drag, may be calculated for any value of n. Further, the variance of the mean difference per drag for N occasions is then s^2/N. In this manner Table 7 was calculated showing that precision can more easily be increased by increasing the number of 'occasions' than by increasing the number of drags per occasion. For example, to double the precision (halve the variance) for 1 drag per occasion, it is necessary to take 15 drags per occasion or to double the number of occasions using 1 drag. Table 7 makes clear that 2–3 blanket drags per plot is sufficient provided the number of 'occasions' (days) is not less than 20. The drags on each occasion must, for reasons given above, be done simultaneously on each plot. The values in Table 7 are not expected to hold exactly for future experiments, but indicate the relative importance of sampling and day-to-day variation.

In Table 6 the totals of nymphs for A and B are 342 and 1302, giving B an average density, ×3·806 higher than A. Since, for this ratio, $\chi^2 = 98{\cdot}02$, D.F. = 32 and $P = 0{\cdot}000{,}000{,}001$ (Fisher, 1941*d*), it is clear (*a*) that the observed heterogeneity in the ratio from day to day is significant, and hence (*b*) that nymphal ticks are very patchily distributed on the ground even when the vegetation cover is apparently uniform in the required respects, and with a sampling unit as large as 300 sq. yd. (the area covered by three drags).

TABLE 7. *The relation of* (1) *the number of simultaneous blanket drags per plot per occasion, and* (2) *the number of occasions of dragging, with the variance of the mean difference of nymphal ticks per drag. Calculated from the data in Table 6 and its analysis of variance in the text*

(1)

No. of drags per occasion	1	2	3	4	5	10	50	100
Variance per occasion of the mean difference per drag	86	63	55	52	49	45	41	40

(2)

No. of occasions		1	2	5	10	20	30	40
Variance of the mean difference per drag for total occasions	With 2 drags per plot per occasion	63	31·5	12·6	6·3	3·1	2·1	1·6
	With 3 drags per plot per occasion	55	27·5	11	5·5	2·8	1·8	1·4
	With 100 drags per plot per occasion	40	20	8	4	2	1·3	1

The totals of male and female ticks in the drags detailed in Table 6 (for nymphs) were:

A		B	
♂	♀	♂	♀
7	6	17	12

The smallness of the totals for the 198 drags is striking when comparison is made with those for nymphs (Table 6). Not more than two males or females per drag were found, frequencies being as follows:

No. of ticks per drag	No. of drags			
	A		B	
	♂	♀	♂	♀
0	191	192	185	186
1	7	6	9	12
2	0	0	4	0

Although the totals for both females and males are higher for bracken bed B than for bracken bed A, the respective '*t*'s' are 1·437 and 1·538 with D.F. 32.

Thus, if reliance had been placed on the blanket counts of either females or males, no significant difference between the plots would have been found.

Discussion

(i) *Sheep versus blanket in the estimation of relative ground population densities of ticks*

Apart from the relation between activity and ground population of ticks, the question arises whether sheep or blanket is preferable in the estimation of relative activities.

The sheep tick has numerous wild and domestic mammalian and avian hosts (MacLeod, 1932, 1934). On any given section of infested grazing, the host potential of a species for ticks may be defined as the product of (1) average individual body surface area suitable for tick attachment, (2) the average area of the grazing covered by the individual in unit time, and (3) the total number of individuals of the species present. The question of comparative host potentials is under investigation, but in view of (1), (2) and (3) above and of the fact that obvious cases of tick spread invariably result from the movements of sheep, the sheep would appear to have by far the highest host potential of the hill fauna. This implies that the sheep is mainly responsible for the ground distribution pattern of the sheep tick. Thus, the natural distribution of ticks on the ground will suffer practically no disturbance by counting the ticks weekly on sheep and leaving them alive *in situ*. With the blanket the ticks are collected to the end of a strip of land 50 × 2 yd. from which point they cannot be redistributed naturally, hence the necessity for taking a 'virgin' strip of land for each drag (see pp. 244, 247).

In the comparison of larger areas, where the character of the vegetation cover generally varies largely, sheep, working constantly over the ground during daylight, will furnish a truer picture of average activity since they sample a larger area probably more randomly than is possible by selective blanketing: in such a case the sheep is to be preferred. Unfortunately, hill-grazing-improvement trials may have to be carried out on plots varying from 1½ to 3 acres because of expense and there are several practical difficulties attached to keeping twenty control sheep in such small areas of unimproved hill land for an adequate length of time. Thus, on small areas the blanket method is easier, but on large varied areas the sheep method is preferable.

There is a further drawback to the use of the blanket, more especially where the plots to be compared are of different vegetation composition, e.g. a grass plot and a bracken plot. The estimation of k would take into account the different frictional effects arising from the nature of the vegetation on the plots, but it would only lead to a better estimate of the total ticks, whether lost or retained, picked up by the blanket. The ticks picked up by the blanket are unlikely to be all the ticks active at the moment on the ground crossed by it. The blanket would only pick up all the active ticks provided they were all on the tips of the plants and the blanket touched all those tips, as might be the case if the tips were level, i.e. on a plane, or a uniform curved surface. Vegetation surface height is always uneven, and the area of the ground crossed which is actually sampled (i.e. tips touched) depends on the relative proportions of the higher and lower components of the vegetation. A bracken bed surface varies according to the time of year. In spring, before the new season's fronds appear, there are scattered 'heaps' of withered fronds of the previous year's crop. To a certain extent, these prevent blanket contact with the rough grasses, low at that time, which are always present in a bracken bed. There follows a transition period, the best time for comparing a bracken bed with a grass plot, during which the withered bracken is settling and the young fronds are growing to the height of the intermingled grasses. Later, the new fronds, growing faster and taller than the grasses, again cause the blanket to 'ride'. When active, ticks rise to the highest point in their immediate vicinity and some will be missed in blanketing according as the higher or lower vegetation is available to them. In a rough grass stretch, free of bracken, the vegetation (tips) surface is also uneven but less so than is generally the case in a bracken bed and it does not vary so much in time. Thus, a lower proportion of active ticks will be missed when blanketing the rough grass type of plot. Hence, on equally densely populated bracken bed and grass plots, blanketing at certain times ought to show a higher density of activity on the grass plot (cf. Table 4 and p. 245). The difficulty in estimating relative densities in such cases is clear and there does not appear to be a practicable method whereby an accurate quantitative estimate of the area of tips surface touched by the blanket can be made. At present, there seems to be no way of surmounting the difficulty except to note the behaviour of the blanket so that it is used for comparison only in those cases where it appears that the proportion of tips surface touched by it is reasonably similar on each plot. This question is less aggravated where plots of the same vegetation composition are being compared.

(ii) *The total density of ticks on the ground*

It may be asked why ground population densities are not compared by some method similar to that for wireworms (Finney, 1941; Yates & Finney, 1942). Cameron (1939, unpublished) found such an approach to the problem impracticable, scarcely observing a single tick in a series of turves from a heavily infested Selkirkshire farm in autumn and winter.

From weekly counts on ten marked sheep, and assuming 7 days as the engorgement period (MacLeod, 1932, 1939*a*), present data show that on the most

heavily infested grazings, stocked at 1 sheep/acre, a ewe feeds at most 1000 female ticks from mid-March to the end of June (spring season of activity). According to MacLeod (1936), a very small proportion of the 1000 will arise from nymphs fed in the early part of the same season. Considering the host-potential of sheep stocked at 1/acre and that the sheep, being to some extent a creature of habit, tends to drop gorged ticks where she will pick them up in their next stage on revisits, it is unlikely that more than a very small proportion of the active females on the ground miss feeding in any season. Nevertheless, it would need a proportion of 4 missing to 1 catching a sheep host to raise the density of female ticks to even 1/sq. yd. per spring season. MacLeod (1939*a*) stated that nymphs are about $\times 2\frac{1}{2}$ more numerous than females on sheep but even this does not improve the position appreciably. The highest single drag count recorded in the course of the present 3 years' work was 53 nymphs. Allowing for as many to be scraped off, this implies 1/sq. yd. for a point in time. A difficulty in using figures 'per sq. yd.' is that they assume uniform distribution of ticks on the ground and this is incorrect. From the practical and statistical viewpoints, the labour of adequately sampling ticks on the ground by examination of random samples of turves is prohibitive, even were a quick and efficient method of extracting tiny ticks from matted vegetation available. The alternative is to cage sample areas against mammals and birds and flag these frequently, but the practical difficulties are enormous.

The study of relative tick activities by sheep or blanket counts as indicative of relative densities seems the only feasible approach to the problem of comparing ground densities.

If the sheep is the mainstay of the tick, the total of weekly counts on sheep (stocked at 1/acre) in a season should furnish a fairly reliable estimate of all females on the ground for that season.

(iii) *The distribution form of tick counts*

The more a sheep moves about in grazing, the more ticks it should encounter. Since on the average hill grazing the nutritious 'bites' are few and far between (most being herbage unpalatable to sheep), a hill sheep has to be very active to maintain a bare existence. Few sheep will *greatly* exceed the necessary minimum activity, but none can go below it. Here probably is part of the explanation for the positive skew in the distribution of tick counts on sheep.

If the individuals of an insect species be distributed randomly and independently of each other on a piece of land, i.e. if there be no tendency even for very localized patchiness, the numbers found in random samples of the area would follow the Poisson law. If individuals lay their eggs in masses, then similar random sampling for larvae almost invariably yields too many samples with no larvae and too few with only one larva—as compared with the Poisson law (Neyman, 1939; Beall, 1940). The reason seems to be the relatively slow dispersal, from the focus of the egg mass, of the larvae in search of food. Thus, if there is one larva in a sample there is likely to be more, and hence the adjective 'contagious' is used to describe such distributions. Neyman (1939) evolved expressions for three types (A, B and C), some of which were successfully fitted to actual larval counts by Beall (1940). Since (1) female ticks show a slight tendency towards random distribution on the ground, (2) they lay their eggs in masses of about 2000, and (3) it is thought that the larva (or any tick stage) moves only a few inches at most in the horizontal plane, it might be expected that larvae should show signs of 'contagious' distribution. Detailed counts are not available, but it was noted that in a group of sheep, the majority carry no larvae, while the remainder may have many. Blanket drags show similar results. Since larvae from one egg mass are concentrated within a small diameter, probably a few inches, hundreds may be picked up at one time by one sheep, and hence will drop off gorged all about the same time. Nymphs, therefore, should also tend to occur in patches though these will be relatively very large as compared with the larval ones. Using Beall's simplification of Neyman's type A 'contagious' distribution with two parameters—the probability that the number, X, of ticks occurring on a given unit area will be n, is given by

$$P(X=n+1)=\frac{m_1 m_2 e^{-m_2}}{n+1}\sum_{k=0}^{n}\frac{m_2^k}{k!}P(X=n-k),$$

starting from $P(X=0)=e^{-m_1(1-e^{-m_2})}$

—theoretical frequencies were included for nymphs in Table 5. Though there is a greater resemblance to this contagious distribution than to the Poisson in case 3 and case 4, the fit is not satisfactory in general. In view of the opinion expressed on p. 244, that 25 yd. drags probably give a truer estimate of actual density of nymphs on the ground than 50 yd. drags, it is interesting that case 3 shows least disagreement with the contagious distribution, this being the only one in which P is greater than 5% ($\chi^2_{[2]}=1{\cdot}63$).

In both counts on sheep and on the blanket, the lower the mean, the nearer do the distributions approach the Poisson type. However patchy the distribution over a field, if activity is so low that only rarely is more than one individual per 'patch' active, it is reasonable to expect an approach to the Poisson distribution. It must also be remembered that the more parameters for a distribution, the closer will the observed data fit. Hence the data for blanketing might be expected to give in general a better fit with the contagious than with the Poisson distribution.

With such a complicated web of factors influencing tick distribution on the ground, the distributions are

inevitably complex. At present, in comparing tick counts, there is no alternative to treating those distributions as normal which, with the qualification already indicated in the text, appears to be reasonable.

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Notes on Thysanoptera found on flax (*Linum usitatissimum* L.) in the British Isles

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The following eighteen species of Thysanoptera Terebrantia have been found on flax in the British Isles: *Melanthrips fuscus* (Sulzer), *Aeolothrips fasciatus* (L.), *Anaphothrips obscurus* (Müller), *Aptinothrips rufus* (Gmelin), *Chirothrips manicatus* Hal., *Limothrips cerealium* Hal., *L. denticornis* Hal., *Stenothrips graminum* Uzel, *Taeniothrips atratus* (Hal.), *T. vulgatissimus* (Hal.), *Thrips angusticeps* Uzel, *T. discolor* Hal., *T. flavus* Schrank, *T. fuscipennis* Hal., *T. major* Uzel, *T. minutissimus* L., *T. physapus* L., *T. tabaci* Lindeman. Each species is described briefly with notes on habits of adults and larvae, place of pupation, number of generations in the year, hibernation, time of occurrence on plants, plants and objects on which found, host plants of larvae and adults, importance to flax, record of locality and collector on flax, distribution, including altitudes, in the British Isles. More species occur in the south than in the north of Great Britain, and species common to both regions usually occur in greater numbers in the south. The insects breed on certain species of crop plants, weeds or trees of arable land. No damage of economic importance to flax by Thysanoptera has been proven in the British Isles, and the flax thrips, *Thrips lini* Ladureau, has not been found. *Taeniothrips vulgatissimus* (Hal.) may breed on flax and its adults, and those of *T. atratus* (Hal.) may cause superficial damage to petals of flowers. *Thrips angusticeps* Uzel and *T. tabaci* Lindeman will probably breed on flax.

During the last few years I have paid particular attention to the Thysanoptera found on flax (*Linum usitatissimum* L.), chiefly in north-east Scotland. I have also received collections for identification from Mr R. Chamberlain of Belfast, Mr W. E. H. Hodson of Reading, Mr J. R. W. Jenkins of Aberystwyth and Mr J. Stapley of Cambridge. Damage of economic importance to flax by Thysanoptera in the British Isles seems unproven, though Mr Hodson and Mr Chamberlain have informed me of suspicious cases.

The flax thrips (*Thrips lini* Ladureau) damages the crop by stunting the plants and by reducing the set of seed, and it may be a serious pest. It is recorded from France (Ladureau, 1877), Germany (Wittmack, 1875), Holland (Doeksen, 1938), Russia, Czecho-Slovakia and Poland. Apparently it has not been found in the British Isles, but there seems no reason why it should not occur here in regions where flax has been grown for many successive years. Doeksen writes that it breeds only on the flax plant, and if this be so, and if the species existed in Britain, the periods when flax growing reached a very low acreage at the end of the nineteenth century and in 1930–1 (Searle, 1940) would cause its disappearance in areas where no flax was grown for some years, unless it was able to maintain itself on escaped flax plants. Evidence is lacking that it can breed on wild Linaceae.

Many different species of Thysanoptera may be found on flax, and it is not practicable to publish a satisfactory key for their identification. It is hoped that the following notes will be helpful to entomologists, both in the field and in the laboratory.

The literature on Thysanoptera is extensive and scattered in many journals. Priesner's (1926, 1927, 1928) *Die Thysanopteren Europas* is invaluable; Uzel's (1895) monograph includes a chapter on the thrips found on flax; Blunk (1923) wrote an admirable account from the economic point of view; Watson's (1923) translation of Karny's key to the genera is useful though especially written for American species; Hinds (1902) gives much useful information; Doeksen (1938) mentions the species found in flax fields in Holland.

Classification

For the purposes of this paper Thysanoptera may be divided as follows:

Suborder 1. Terebrantia. Female with an ovipositor used for inserting the smooth, delicate-shelled egg in the superficial tissue of plants. Male with the end of the abdomen bluntly rounded. Wings bearing microscopic hairs on the surface and bristles on the veins; the forewing with a marginal vein and at least one longitudinal vein reaching the margin near the apex of the wing.

Family Melanthripidae. Ovipositor curved backwards. Wings broad and rounded at tips. Antennae 9-segmented with all the segments freely movable. Labial palps 2-segmented. Two larval instars. Larva active, with 7-segmented antenna. Probably one prepupal and one pupal instar.

Family Aeolothripidae. Ovipositor curved backwards. Wings broad and rounded at tips. Antennae 9-segmented with the terminal 5 segments united. Labial palps 4-segmented. Egg bean-shaped. Two larval instars. Larvae with 7-segmented antennae; active, feeding on the juice of plants and predacious on other small insects including Thysanoptera. Prepupal and pupal instars in tough silken

cocoon. Prepupa stated to carry its antennae curved backwards over the head.

Family THRIPIDAE. Ovipositor curved forwards. Wings narrow and pointed at the tips. Antennae 6–9-segmented. Eggs elongated ellipse or kidney shaped. Two larval instars which are generally yellowish, feeding on plant juices. One prepupal and one pupal instar do not feed, normally quiescent, but capable of walking. Antennae of prepupa are practically unsegmented and project straight in front of the head: in the pupa they are bent backwards over the head and thorax.

SUBORDER 2. TUBULIFERA. Female without ovipositor. The 10th abdominal segment in both sexes is tubular. Wings without microscopic hairs on the surface or a long series of bristles on the veins, but fringed with long cilia. Forewing with one short median vein. Eggs, hard-shelled and often sculptured, are deposited on the surface of plants. Two larval instars which are often brightly coloured or banded, feed on juices of plants, or more rarely, those of insects. One prepupal and two pupal instars.

Family PHLAEOTHRIPIDAE. With the characters of the suborder.

All Thysanoptera of which I have records from flax in the British Isles belong to the Terebrantia. On the European Continent various Tubulifera, particularly certain species of *Haplothrips*, are common in sweepings from herbage. It seems likely that they would occur in sweepings from herbage, including flax, in the south-eastern counties of England, from which there are practically no records of Thysanoptera. Uzel (1895) and Doeksen (1938) record *Haplothrips* from flax in Europe.

Since Thysanoptera are Insecta Paurometabola, their immature feeding stages are generally called 'nymphs' in English entomological text-books, in which the word 'larva' is applied to the immature feeding stages of Holometabola. European continental authors often use the term 'nymph' for the thysanopterous prepupa and pupa. I use the term 'larva' for the immature feeding instars of Thysanoptera, and following Priesner (1926, 1926–8) the Roman numerals I and II after larvae, and in the Tubulifera after pupae to denote the instar.

The wings vary considerably in size in certain species, ranging from full development to minute scales, and they may be absent. It is convenient to use the terms *macropterous* for specimens with fully developed wings, *hemimacropterous* for those with wings intermediate between full development and scale-size, *micropterous* for those with scale wings and *apterous* for those without wings.

COLLECTION AND PRESERVATION

Flax may be examined for Thysanoptera by tapping the plants sharply over a sheet of stiff paper on to which the insects fall. The insects may be picked off the paper with a fine paint brush moistened with 50% methylated spirit from a tube in which the specimens are placed. After 2–3 days the spirit should be decanted from the tube and replaced by 80% ethyl alcohol to preserve the insects. The 50% spirit macerates and makes adults comparatively easy to spread out on the mount. The tube containing the thrips should also contain a paper labelled in pencil with the name of the plant or object on which the insect was found, locality, date of capture and name of collector. The thrips from each species of plant or other object should be collected and kept in a tube by themselves. For microscopic examination adults and immature instars may be placed in water, dilute alcohol or glycerine, glycerine jelly or one of the mounting media such as Faure's fluid or Swan's (1936) modification of Berlese's medium. For permanent mounts a resinous medium like Sira mountant or Canada balsam is the most satisfactory.

DISTRIBUTION OF THYSANOPTERA IN THE BRITISH ISLES

Very little is known about the distribution of the different species of Thysanoptera in the British Isles, where there are about 182 named species, but from records and personal observations more species occur in the south than in the north of Great Britain. The greater number of species in the south is correlated with larger number of species of plants established there. The distribution from east to west is even less known, though North Ireland (my collections and Chamberlain's specimens) has many of the common species of Britain and, apparently, no species peculiar to itself. In Skye and the Outer Hebrides the fauna is like that of the mainland of the same latitude, except that there are fewer species and smaller numbers. In Shetland the fauna is like that of the north mainland, but again with fewer species and scanty numbers of each species. In all these smaller islands, the chief factor influencing the numbers of species and specimens of Thysanoptera, in addition to climate and vegetation, is probably intensive grazing by animals, particularly sheep. Thysanoptera will almost certainly be found on flax throughout the British Isles and frequently in abundance during June–September.

BIONOMICS, HABITS, HOST-PLANTS

Every species of the Thysanoptera Terebrantia likely to be found on flax has its life history linked with that of one or more species of flowering plant, usually an annual. Most species that occur on flax do not breed on it, nor are they likely to harm it economically by feeding on it. They are the common denizens of neighbouring crops of Gramineae, crucifers, weeds or trees of arable land.

Flax can probably be grown in most parts of the British Isles and it occurs as an escape in various

places: it is grown for fibre widely in Great Britain and North Ireland, growth for seed being more restricted in area. It is a potential host for feeding Thysanoptera throughout the life of the plant. The plant is normally green for about 3–4 months (Searle, 1940; Stirling, 1941), but with the differences in the time of sowing, weather and geography, green plants may be found somewhere in the British Isles from mid-April to mid-October. It is probable that other species of *Linum* or of closely related genera would also serve as hosts for species feeding on flax, and the wild and cultivated species of Linaceae should be considered potential hosts. The wild species included in the British Flora are *Linum catharticum* L. abundant in Britain, L. *bienne* Mill. (*angustifolium* Huds.) southern and western England, *L. perenne* L. localized in some eastern counties of England, and *Radiola linoides* Roth. (*millegrana* Sm.) generally spread over Britain and very abundant in some localities though scarce in others. There are various garden species of *Linum*. No Thysanoptera have been recorded from these plants in the British Isles, probably because they have never been examined for these insects.

The previous crop, neighbouring crops, weeds, methods of cultivation, and probably sometimes migrations of thrips, affect the numbers of species and specimens of thrips on flax. Plants on the margin of a field are likely to harbour more than those in the centre. Some species appear earlier than others, some are univoltine and appear on plants for only a few weeks, whilst others have two to three generations in the year and occur for many weeks on plants, with much overlapping of the generations. In most species the proportion of sexes varies: females are usually found in much greater numbers than males and males are unknown in at least one species. Males are usually smaller and in some species paler than females. Field observations indicate that all species likely to occur on flax thrive during dry weather when there is sufficient water for the plant to grow (cf. Wardle, 1927). Thrips may be particularly active and numerous during spells of dry, thundery weather. When catching specimens in the field the heat of the beating tray and perhaps also the bright light on a hot day makes normally sluggish adults and larvae very active, and normally active adults more inclined to jump and fly if they are capable of either or both these activities. Continuous rain is harmful to thrips partly through its mechanical action in washing them off plants.

The extent to which thrips can injure flax was described and figured by Doeksen (1938). The mouthparts of adults and larvae penetrate the cuticular layer of cells of plants and only one or two layers below this (cf. Wardle & Simpson, 1927). When much feeding has taken place on leaves, petals or green stems the surfaces are marked by a silvery sheen, due largely to air occupying the cell cavities, from which the insects have removed the normal contents of protoplasm and fluid. Certain species mark the attacked surface with spots of faeces which often become dark coloured.

Some species breed on one species of plant only, some on members of the same genus or of closely related genera of plants, whilst others breed on many genera in a family or even in different families. Besides those plants on which a species of thrips may breed, adults of certain species feed also on other plants and they may be found on many different species of plants and objects. A species of plant that serves for rearing larvae of a species of thrips I call a *host plant* of that species. The plant or object on which the insect is found may be called the *finding place*. Sometimes the finding place is also the host plant, but too often authors name the plant on which an insect happens to have been found the host plant of the insect, without any proof that the insect feeds or breeds on that plant. Amongst British species four have been named after plants which are not their host plants. Even when the natural food of a Thysanopteron is plant juice the species may under certain conditions drink human blood or blood plasma. Bailey (1936) discussed a number of instances of Thysanoptera attacking man, and I have experienced bites from species that normally feed on plants.

Enemies of thrips on flax

Red parasitids (Gamasidae) are predacious on adults and larvae in the open during June–Sept. (cf. MacGill, 1939). Immature red mites often attach themselves to adult thrips in spring and in smaller numbers in autumn to the thin cuticle between the thoracic sclerites and those of the first abdominal segment. I have no evidence of any harm done to the insect. Small spiders sometimes kill thrips. *Aeolothrips* larvae and adults are predacious on adults and larvae of other Thysanoptera. I have not found parasitic Hymenoptera in the species found on flax. A nematode parasitizes *Aptinothrips* (Sharga, 1932). Fungi developing in the body cavity kill many adults and larvae in autumn.

Thysanoptera found on flax

The Thysanoptera described below have been found (marked with an asterisk) or are likely to be found on flax in some parts of the British Isles. The coloration described is that of fully mature adults and of full fed II instar larvae. Young adults, I instar larvae and newly emerged II instar larvae are paler. Overwintering adults are usually darker than the summer generation. Plants and insects appear about 3–4 weeks later in spring in north-east Scotland than in southern England, so a species of thrips that is common to both regions may have one or two generations less in the year in the north-east than in the south.

THYSANOPTERA

Suborder **Terebrantia**

Family MELANTHRIPIDAE

*Melanthrips fuscus** (Sulzer). Adults dark brown with many long hairs on head and thorax; wings brown, with rounded tips. Larvae yellow. Adults and larvae active. Pupation probably in soil, possibly in cocoon. One generation in year in north-east Scotland. Probably hibernate in soil as larvae which pupate in spring. ♀♀ occur May–Sept., ♂♂ May–July, larvae June–Sept. Adults and larvae often together in fair numbers on host plants which include *Brassica Sinapis*, *Brassica* spp., particularly young or flowering plants, *Poterium Sanguisorba*, *Galium Aparine*, *Pisum sativum*. More ♀♀ than ♂♂ occur scattered on other species of plants. On flax 1 ♀ Norfolk (Stapley), 1 ♀ Pembrokeshire (Jenkins). Probably no economic importance to flax. Common in southern England and reaching north-east Scotland where it is scarce. Sea level to at least 600 ft. altitude. Genus has not been found in north Ireland. *Melanthrips* spp. occur in southern England and some other species are likely to occur on flax, particularly in the vicinity of flowering *Brassica* spp.

Family AEOLOTHRIPIDAE

*Aeolothrips fasciatus** (L.). Most winged specimens of the genus are recognizable in the field by the naked eye from the two distinct, white crossbands on the wings contrasting with the dark brown body. Larvae yellowish. Adults and larvae run actively. Pupation probably in tough silken cocoon in soil. Probably two generations in year in southern England. Probably hibernate in soil as larvae which pupate in spring. ♀♀, ♂♂ occur May–Sept., larvae June–Sept. Adults of both sexes and larvae found, usually scattered, sometimes numerous on many different species of wild and cultivated plants. Host plants of larvae uncertain since this species has been confused with at least one other species found in southern England. Adults and larvae seem to imbibe juices of plants as well as being predacious on larvae and adults of other Thysanoptera. On flax 9 ♀♀, 4 ♂♂ Norfolk (Stapley). Likely to be beneficial to plant because of predacious habits. Common in southern England and reaching north-east Scotland where it is rare. Sea level to at least 400 ft. altitude. Genus has not been found in north Ireland. *Aeolothrips* spp. common, at least in southern half of England, where some other species are bound to occur on flax.

Family THRIPIDAE

*Anaphothrips obscurus** (Müller). Breeds on certain Gramineae. Females yellow to pale brown, macropterous or micropterous. Males unknown. Larvae white or very pale yellow, usually with a green tinge due to chlorophyll in contents of gut showing through body wall. Females, larvae not very active. Pupation amongst vegetation or in soil. Two generations in year in north-east Scotland. Macropterous and micropterous ♀♀ hibernate in soil or amongst herbage. Some may be found on plants throughout the year; most numerous June–Sept. Larvae May–Dec., most numerous June–Sept. I have found ♀♀ on more than 32 Gramineae spp. and on 9 species of other families of flowering plants. Most favoured host plants are *Holcus lanatus* and *H. mollis*, apparently less favoured are oats, barley, wheat, *Lolium* spp., *Agrostis palustris*, *A. tenuis*, *Arundo phragmites*, *Agropyron repens*, *Aira* spp., *Dactylis glomerata*, *Poa annua*. May be a pest of cereals in Sweden (Johansson, 1938). Hinds (1900) figures its instars as *Anaphothrips striata* (Osb.) and describes damage to grass in Massachusetts. Probably harmless to flax. One macropterous ♀ Pembrokeshire (Jenkins). Widely distributed in British Isles and most abundant in warmer parts. Breeding from seashore to altitude of at least 1200 ft., and occurring at least as high as 1700 ft.

*Aptinothrips rufus** (Gmelin). (I do not include *A. nitidulus* Hal. in this species.) Breeds on certain Gramineae. Yellow, apterous, ♀♀ numerous, ♂♂ scarce. Larvae pale orange. Adults and larvae not very active. Pupation in herbage. 2–3 generations in year in north-east Scotland with much overlapping of the generations. Adults of both sexes and all immature stages may be found on plants throughout the year. ♀♀ and larvae most numerous July–Sept. Some hibernation in soil when grass is dead. Finding places not so varied as for some other species, probably owing to lack of wings of adults. Host plants include *Agropyron repens*, *Agrostis* spp., *Alopecurus pratensis*, *Ammophila arenaria*, *Anthoxanthum odoratum*, *Arrhenatherum elatius*, *Cynosurus cristatus*, *Dactylis glomerata*, *Deschampsia caespitosa*, *D. flexuosa*, *Digraphis arudinacea*, *Festuca rubra*, *F. ovina*, *Glyceria maritima*, *Holcus lanatus*, *H. mollis*, *Lolium* spp., *Molinia coerulea*, *Nardus stricta*, *Phleum pratense*, *Poa annua*, *P. nemoralis*, *P. pratensis*, oats, wheat, barley and less frequently, *Lotus* spp., *Vicia Cracca*, *Cytisus scoparius*, *Carex* spp., *Juncus* spp. Probably harmless to flax. Pembrokeshire 1 ♀ (Jenkins), ♀♀ Co. Down, Aberdeenshire (Morison). Apparently distributed throughout British Isles, ranging from grass which is submerged by normal high tide to grass at least 2600 ft. above sea-level. Sharga (1933) discussed its life history and some parasites, Speyer (1935) its systematics. Red Parasitids are predacious on it during June–Sept.; immature red mites attach themselves to females in spring and again in autumn; Sharga (1932) described a parasitic nematode.

Aptinothrips stylifer (Trybom) resembles *rufus* closely, has similar habits, host plants and distribution, but is generally scarcer. Almost certainly it will be found on flax when this is mixed with grass.

Baliothrips dispar (Hal.), *Odontothrips* spp., *Oxythrips* spp. are likely to occur on flax growing in the vicinity of the host plants of the respective species. They are not likely to harm flax.

*Chirothrips manicatus** Hal. Breeds on certain Gramineae. Dark brown, broad, dorso-ventrally flattened, ♀♀ macropterous, rarely micropterous or apterous, ♂♂ micropterous. Larvae lemon to orange-yellow, broad, flattened, very short antennae. Adults and larvae sluggish. Pupation probably in soil or under vegetation. Apparently one generation in year in north-east Scotland (cf. Morison, 1928). Hibernation by ♀♀ and perhaps by larvae. Females occur on plants May–Dec., most during July–Aug., ♂♂ July–Oct., larvae July–Aug. Both sexes often abundant. Winged ♀♀ liable to migrate July–Aug. Larvae very difficult to find. Finding places chiefly grass and cereals. Host plants *Deschampsia flexuosa*, *Dactylis glomerata*, and other grass species. Probably harmless to flax. Pembrokeshire 1 ♀ macropterous (Jenkins), Norfolk 2 ♀♀ macropterous (Stapley), Aberdeenshire 2 ♀♀ macropterous (Morison). Widely distributed in Britain from sea-level to at least 1000 ft.

Frankliniella intonsa (Trybom) Brown, ♀♀, ♂♂ macropterous, with two long hairs near each anterior angle of the pronotum as well as a pair near each posterior angle. Larvae orange. Adults active, may jump, larvae fairly active. Pupation probably in soil or under vegetation. Probably 2 generations in north-east Scotland. Hibernation probably by ♀♀. Occur on plants, ♀♀ May–Nov., ♂♂ June–Oct., larvae July–Sept. I have found the insect on forty-seven species of flowering plants. Host plants are *Orchis maculata* (Speyer & Parr, 1941), *Calluna vulgaris*, *Ononis arvensis*, *Rosa* spp., and other plants. So widely spread and numerous in southern England that it is bound to occur on flax, which it probably does not harm. Scarce in north-east Scotland except on certain moors. It has not been found in north Ireland. Ranges from sea level to at least 600 ft.

Frankliniella tenuicornis (Uzel). Both sexes macropterous, yellow to dark brown, jump actively. Pupation on host plant and probably in soil. Hibernation by ♀♀ and ?♂♂. Occur on plants ♀♀ June–Nov., ♂♂ July–Aug., larvae June–Aug. Generations 2 in north-east Scotland, probably 3 in southern England (cf. Johansson, 1938). Widely distributed in Britain from sea-level to at least 300 ft. Breeds, often abundantly, on barley, oats, wheat and probably certain grasses. Almost certain to occur on flax and not to harm the plant.

Limothrips cerealium Hal.* Corn thrips. Dark brown, elongated, dorso-ventrally flattened, ♀♀ macropterous, ♂♂ apterous. Larvae pale orange yellow, flattened. Adults and larvae sluggish. Pupation amongst vegetation. Two generations in north-east Scotland, though Morison (1928) for the north-east and Sharga (1933) for the east of Scotland considered that there was only one generation. The insect is essentially a corn thrips. If the corn is cut in August there is time for only one generation, but if as often happens the oats are not cut till the end of September or late in October, there is time for a second generation. Females hibernate and may be found throughout the year. They appear in Apr.–May on grass and migrate during June–July to cereals on which they breed, reaching maximum numbers during harvest in August, when they may migrate in large numbers. Males found June–Oct., larvae May–Nov. I have found the winged ♀♀ on twenty-nine species of Gramineae and on forty-eight other species of flowering plants, besides hibernating in wood or bark, on house walls, amongst dead flies, lepidopterous cocoons, and *Cryptoccus fagi* (Bärens.) on beech. The apterous males are restricted to Gramineae and plants adjacent to them in the field. Host plants cereals and a few grasses. Harmless to flax. Pembrokeshire 3 ♀♀, 2 ♂♂ (Jenkins), Norfolk 6 ♀♀ (Stapley), Aberdeenshire ♀♀ (Morison). Widely distributed, often abundant in British Isles, ♀♀ from sea-level to at least 1200 ft. Commoner in south than in north.

*Limothrips denticornis** Hal. Chief corn thrips of many European countries. Larger than *L. cerealium*, 3rd antennal segment with a lateral spur. Larvae white. Like *cerealium* in life history, habits and host plants, but usually much scarcer in numbers in Britain where it seems to favour barley. Sea level to at least 600 ft. Harmless to flax. Pembrokeshire 3 ♀♀ (Jenkins), Norfolk 5 ♀♀, perhaps from weeds in flax field (Hodson).

*Stenothrips graminum** Uzel. An oat thrips. Both sexes macropterous, yellowish grey to brown, cylindrical, hairs pale coloured. Larvae yellow, with eight strong, sharp-pointed processes on the posterior dorsal margin of the 9th abdominal segment. Adults, larvae active. Pupation in soil (Kolobova, 1926). Probably two generations in southern half of England and Wales (cf. Kolobova, 1926). Hibernate as larvae (Kurdjumov, 1913). I have found ♀♀ May–Oct., ♂♂ June–July, larvae July. The records of Bagnall and Williams for this species in Britain are within these dates. My captures were made during brief visits at various times over 4 years to different places in southern England. Almost certainly the species will occur on vegetation for longer periods than those given. I have found it on eleven species of flowering plants. According to Kolobova it is a pest of oats and breeds only on them and *Avena fatua* in South Russia, but it is likely that in England it also breeds on *Lotus corniculatus*, *Trifolium* spp. and certain grasses. From Stapley I received five larvae II from flax in Norfolk, also 10 ♀♀, 17 ♂♂ from another locality, so there is a possibility that it breeds on flax. Pembrokeshire 1 ♀, 1 ♂ (Jenkins). It is common on flax in Holland (Doeksen, 1938). Bagnall recorded the species from Berks, north Devon and Oxford, and I have a slide of his specimens from Bridlington, Yorks. Williams records it

from Cambridge and Oxford. I have found it, often numerous, in Beds, Berks, Bucks, Cambridge, Middlesex, Glos, Surrey, Wilts, but not in Scotland or north Ireland. Sea level to altitude of at least 400 ft.

*Taeniothrips atratus** (Hal.). Both sexes dark brown or almost black, macropterous with a single narrow white cross band on the wings to the naked eye; active, jump. Larvae orange. Pupation amongst herbage or in soil. Two generations in the year in north-east Scotland (Morison, 1929). Hibernation by ♀♀, and some may be found throughout the year on plants. They appear in numbers on herbage in Apr.–May. Males appear in mid-June amongst the progeny of overwintered ♀♀, reach maximum numbers in August and disappear by the end of November. Larvae June–Oct. I have found the species on 202 species of flowering plants. Host plants are species of *Spergula*, *Cerastium*, *Stellaria*, *Capsella*, *Statice* and other genera. Both sexes may occur in hundreds on flowers, white boards, or other pale-coloured dry objects like straw or paper in the field during Aug.–Sept. They will feed on the petals of flax flowers and cause some bleaching, but I have not noticed economic damage to the plant. Pembrokeshire 11 ♀♀, 28 ♂♂ (Jenkins), Co. Tyrone 9 ♀♀, 5 ♂♂ (Chamberlain), ♀♀, ♂♂ Co. Down, Aberdeen (Morison). Distributed throughout British Isles, more numerous in north than south Britain. Sea-level to at least 1100 ft.

*Taeniothrips vulgatissimus** (Hal.). Like *T. atratus* in appearance and habits (Morison, 1929). Larvae yellow. Pupation on plant or in soil. Two and possibly a partial third generation in north-east Scotland. Females hibernate and some may be found throughout the year on plants. They appear in numbers on plants in Apr.–May. Males found June–Nov., larvae May–Oct. I have found the insect on 197 species of flowering plants. Overwintered ♀♀ oviposit chiefly in *Salix* spp. Sexes occur in about equal numbers in the two succeeding generations, but ♂♂ disappear before winter. Second generation reared on umbellifers, *Brassica* spp., *Barbarea*, *Cochlearia*, *Lepidium*, apple, pear, *Crataegus*, *Spirea*, *Rumex*, *Ulmus*, *Vicia Cracca*, *Lathyrus*, *Valeriana*, *Stellaria*, *Linum usitatissimum*. I have found the species breeding on flax without causing serious injury. Adults may be associated with those of *atratus* in harming flax flowers. Pembrokeshire 120 ♀♀, 2 II (Jenkins), Co. Tyrone 51 ♀♀, 2 ♂♂, 3 I, 15 II (Chamberlain), ♀♀, ♂♂, II, Co. Down, Kincardine, Aberdeen (Morison). Distributed throughout British Isles, more numerous in north than in south Britain. Sea-level to at least 2500 ft. Adults and larvae of *T. atratus* and *T. vulgatissimus* have enemies in predacious red mites and *Aeolothrips* during June–Sept. and in fungi during Sept.–Nov. Adults in the later stages of attack by fungus are usually almost black in colour, with all parts of the body and limbs full of dark spores of fungus. The genera *Thrips* and *Taeniothrips* are very closely related. In the southern half of England the Thysanoptera most numerous on many different flowering plants are *Thrips fuscipennis* Hal., *T. flavus* Schrank, *T. major* Uzel and *T. physapus* L. In north-east Scotland the place of the *Thrips* is taken by *Taeniothrips atratus* (Hal.) and *T. vulgatissimus* (Hal.), though all except one of the species of Thysanoptera and most of the flowering plants are common to both parts of the country.

*Thrips angusticeps** Uzel. Both sexes dark brown, macropterous or micropterous, fairly active. Larvae bright yellow to orange. Pupation in soil (Buhl, 1934). Probably two generations in southern England (cf. Buhl, 1934). Apparently hibernate as micropterous ♀♀, ♂♂ (Buhl, 1934). I have found macropterous ♀♀, June–July, micropterous ♀♀ Apr.–June, micropterous ♂♂ May–June, larvae in July. The dates recorded by Bagnall are within this period. Williams found micropterous ♀♀ Apr.–May. My captures were made during brief visits at various times over 3 years to different parts of southern England. Almost certainly the species will occur on vegetation for longer periods than those stated. Found on many different species of flowering plants. Host plants are kohlrabi, cabbage, Brussels sprouts, mustard, *Galium*, *Mollugo*, *Lepidium draba*, and perhaps swedes, beet, peas, oats and flax. Uzel (1895) records it from flax, Van Eecke (1922) associated it with injury to flax, but Doeksen (1938) after reviewing their papers, states from his experiments that it is not economically important to flax. It is apparently attracted to flax and/or weeds in flax fields for Van Poeteren (1930, 1931) and Doeksen (1938) say that it may harm beet, peas or oats grown after flax in Holland. Norfolk 4 ♀♀ macropterous (Stapley), 2 ♀♀ macropterous (Hodson). Bagnall recorded the species from Berks, Surrey, Sussex; Williams from Cambridge; I have found it in Middlesex, Bucks, Berks, Cambridge, Wilts and in a restricted part of Aberdeen City, and it has been sent to me for naming from Beds, Bucks, Cambridge, Kent, Norfolk and Cardigan. Sea-level to at least 400 ft.

*Thrips discolor** Hal. Females macropterous or micropterous, abdomen dark brown contrasting with the yellowish head, thorax and legs. Males micropterous, yellow. Larvae pale yellow. Adults and larvae rather sluggish. Pupation probably amongst grass. Micropterous ♀♀ hibernate. Probably two generations in north-east Scotland. I have found ♀♀ macropterous June–July, micropterous July–Jan., micropterous ♂♂ July–Sept., larvae August. The capture of this species in the British Isles has not been recorded since Haliday described the insect in 1836. The insect has been found in various European countries, usually amongst grass and herbage in marshy localities, but the larvae and host plants were never recognized. The host plants are *Holcus lanatus* and *H. mollis*. Co. Down 1 ♀ macropterous (Morison) on flax, to which the species is probably harmless.

I have found the insect in Middlesex, Aberdeen, Kincardine, Moray, Co. Down, at altitudes of 100–600 ft. above sea-level.

*Thrips flavus** Schrank. Macropterous, ♀♀ orange, ♂♂ much paler yellow; fairly active; larvae pale yellow. Pupation in soil. Two generations in north-east Scotland, probably three in southern England. Females hibernate, may be found all the year round on herbage, most numerous July–Sept.; ♂♂ June–Oct.; larvae June–Sept. I have found the insect on ninety species of flowering plants, of which the most favoured hosts are *Lonicera Periclymenum*, *Ulex europaeus*, *U. nanus*, apparently less favoured are *Senecio Jacobaea*, Michaelmas daisy, *Sambucus nigra*, *Salvia* sp., *Verbascum* sp., *Rubus fruticosus* (agg.), *Malva moschata*. On flax Pembrokeshire 3 ♀♀ (Jenkins), Aberdeen 2 ♀♀ (Morison). Probably harmless to flax. Distributed throughout British Isles, more numerous in south than in north Britain. Sea-level to at least 600 ft. Recorded as the most numerous Thripid in some European countries. I have no evidence to corroborate the statement by Blattny & Balacek (1924) that it preys on Tetranychid mites in hops in Czecho-Slovakia. Their determination of the species may be wrong, and they may be dealing with a *Scolothrips*.

*Thrips fuscipennis** Hal. Rose thrips. Macropterous, ♀♀ brown, ♂♂ usually paler brown. Larvae yellow. Adults and larvae fairly active. Pupation on vegetation or in soil. Two generations in north-east Scotland, probably three or four in southern England. Females hibernate. Almost certainly some could be found on plants all the year round. I have found them Mar.–Oct. in southern England and June–Oct. in north-east Scotland, ♂♂ June–Oct. in southern England, June–Sept. in north-east Scotland, larvae May–Sept. in southern England, June–Sept. in north-east Scotland. In hothouses in north-east Scotland I have found ♀♀ Feb.–Oct., ♂♂ June–Sept., larvae May–Sept. I have found the insect on 154 species of flowering plants. Host plants include rose, apple, peach, strawberry, *Rubus*, *Senecio*, *Achillea*, *Carduus*, *Aster*, *Phaseolus*, *Pisum*, *Althea*, *Ulex*, *Polygonum*, *Chemopodium*, *Asparagus*. On flax to which it is probably harmless, Co. Tyrone 2 ♀♀ (Chamberlain). Often abundant in England, Wales, north Ireland, and Scotland up to the end of the southern half of Kincardine, and from there northwards it is scarce, usually occurring on fruit trees in hothouses or in gardens. Sea-level to at least 700 ft. Ahlberg (1924) described its life history in Sweden; Speyer (1934) and Priesner (1934) discussed its systematics; Speyer (1938, 1939) described its habits in glasshouses in England.

*Thrips major** Uzel. Macropterous, both sexes very like *T. fuscipennis*, but generally paler coloured. Frequently associated with *fuscipennis* on many different species of flowering plants. Life history probably like that of *fuscipennis*. The species seems widely distributed in southern England where it is often numerous on flowers, but it is not as abundant as *fuscipennis*, and I have no records of it from Scotland. I have found ♀♀ Apr.–Sept., ♂♂ July–Sept., larvae May–Sept. Sea-level to at least 400 ft. Probably harmless to flax on which I found 1 ♀ in Co. Down.

*Thrips minutissimus** L. Both sexes, macropterous, yellow to dark brown. Larvae pale yellow. Adults, larvae fairly active, adults capable of jumping. Pupation probably in soil. Apparently one generation in Britain and other European countries. Probably hibernate as larvae in soil or vegetation. I have found ♀♀ Apr.–July, ♂♂ Apr.–May, larvae May–June, and the species on eighteen species of flowering plants. Hosts are *Quercus* and *Crataegus*. Priesner (1927) gave a long list of plants on which adults have been found in Europe and a shorter list of the plants on which larvae occurred. From my observations *Quercus* is the most favoured host, and adults on their way to it in spring after hibernation (as larvae) in the soil may alight in large numbers on neighbouring vegetation. Probably harmless to flax, 1 ♀ Pembrokeshire (Jenkins). Widely distributed in southern England and extending to at least southern Sutherland. More numerous in the south than in the north of Britain. Has not been found in north Ireland. Sea-level to at least 400 ft.

*Thrips physapus** L. Both sexes macropterous, dark brown, fairly active, capable of jumping. Larvae pale yellow. Pupation probably in soil. Two generations in north-east Scotland, probably three in southern England. Hibernation by ♀♀ (Uzel, 1895) and probably also by ♂♂ (Van Eecke, 1922) since sexes appear together in spring. I have found ♀♀ Apr.–Sept., ♂♂ May–Sept., larvae June–Sept., and the insect of more than twenty-four species of flowering plants in southern England, where, as in many European continental countries, it occurs on many different species of plants of different families; but in north-east Scotland I have found it only on host plants or a few other species of Compositae. Host plants are *Taraxacum* and, rarely, *Hypochoeris*. On flax to which it is probably harmless, 4 ♀♀ Pembrokeshire (Jenkins). Ozols (1930) records it on flax in Latvia. Widely distributed in the British Isles and much more numerous in the south than in the north. Sea level to at least 600 ft. It does not extend far up the hills in northern Scotland, but Priesner (1927) stated that it reaches the snow line in the Alps.

*Thrips tabaci** Lindeman. Onion thrips. Macropterous, ♀♀ yellow to dark brown, ♂♂ paler coloured and much scarcer than ♀♀. Adults and larvae fairly active and adults capable of jumping. Pupation on plants or in soil. Two generations in north-east Scotland, probably 2–3 in southern England. Frequently found in glass houses where there may be many generations in the year. According to Webster (quoted from Hinds, 1902) ♀♀, larvae and pupae hibernate in Ohio. I have found ♀♀ most numerous July–Sept., though some occur on plants in the open

all the year round, ♂♂ usually scattered, sometimes in numbers June–Sept., larvae May–Nov. and one specimen in January, and the insect on 152 species of flowering plants. Larvae may be bred on many species of flowering plants (belonging to several different families) to which they are more or less harmful, and the adults, especially ♀♀ since ♂♂ are very scarce, probably feed on all these and many other species of plants, some of which they may harm. The insect's economic importance throughout the world is indicated by the annual number of references to it in the *Review of Applied Entomology*. Practically cosmopolitan in distribution, it has been called the onion, potato or tobacco thrips depending on the crop attacked. In many cases it is probably forced to attack the crop through the method of cultivation leaving no weeds or other species of cultivated plants as larval hosts in the vicinity of the attacked crop. In Britain it is associated essentially with certain species of Compositae, breeding on *Achillea millefolium*, *A. Ptarmica*, *Senecio Jacobaea*, *S. viscosus*, and many cultivated *Senecio* spp., *Solidago* spp., *Matricaria* spp., *Aster* spp., *Artemisia vulgaris*, *Hypochoeris radicata*, and cultivated chrysanthemum and cineraria. Other larval hosts are *Anthyllis*, *Lotus*, *Lathyrus*, *Aubretia*, *Cheiranthus*, *Cochlearia*, *Althea*, *Armeria*, *Asparagus*, *Campanula*, *Dianthus*, *Digitalis*, *Galium*, *Nepeta* cultivated spp., *Plantago maritima*, *Spergularia rubra*, *Salvia*, *Sedum*, *Triglochin maritimum*, *Teucrium Scorodonia*, and in hothouses peach, plum, cucumber, *Opuntia*, *Oxalis*, *Tradescantia*. Uzel (1895) in Bohemia and Priesner (1927) in Austria stated that it has a preference for Solanaceae and Umbelliferae: this is contrary to my observations of its preferences in Britain. It is known to breed on potato and tomato plants, but I have not found it doing so, however I have had very few opportunities of beating wild Solanaceae for it in England and these plants only occur as rare escapes in north-east Scotland. I have captured thousands of Thysanoptera from Umbelliferae in England and Scotland, but *Thrips tabaci* occurred only as solitary females amongst them. It probably breeds on flax without harming it economically. Norfolk 23 ♀♀ (Stapley), Pembrokeshire 12 ♀♀ (Jenkins), Co. Down 2 ♀♀, Aberdeenshire 3 ♀♀ (Morison). Distributed widely in the British Isles. Sea-level to at least 1200 ft. Generally occurs scattered, but may be numerous on larval host plants, not found in great numbers like the flower-haunting *T. fuscipennis*, *T. flavus*, *Taeniothrips atratus* and *T. vulgatissimus*.

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Biological methods of determining the insecticidal values of pyrethrum preparations (particularly extracts in heavy oil)*

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(With 11 Text-figures)

Pyrethrum extracts in non-volatile oil carriers are effective insecticides in the field because they act as a direct spray killing the insect and also form a toxic film over which the insect crawls: it is necessary to study both these effects for a complete laboratory assessment of toxicity. Suitable laboratory techniques and methods for the assessment of results are described together with an account of experiments on the effect of various factors on the insecticidal efficiency of pyrethrum-in-oil preparations, both as direct sprays and as toxic films.

INTRODUCTION

Potter (1935, 1938) devised a technique for the control of insect pests in houses and warehouses by means of a pyrethrum extract in a heavy non-volatile petroleum oil. This method has proved useful in the protection of foodstuffs in storage and it is probable that it may be of value in other fields of control. It is thus urgent that pyrethrum preparations should comply with a specified standard, and that a means be available for evaluating them and alternative preparations.

Chemical assay alone does not necessarily give a satisfactory estimation of toxicity, and two problems need resolving: (1) the preparation of a solution of pyrethrum extract in oil the composition of which

* EDITORS' NOTE.—Recent work on the use of pyrethrum-in-oil insecticides emphasized the need for satisfactory means for their evaluation and standardization. Chemical determination of the content of pyrethrins may not by itself always give a reliable measure of toxicity, and a standard biological method of evaluation is essential. Early in 1941, discussions between representatives of research laboratories and industrial firms interested resulted in the formation of a small committee under the chairmanship of Dr F. Tattersfield to consider the best means of dealing with this problem. As a result of the work of this committee, it was agreed that the Pest Infestation Laboratory of the Department of Scientific and Industrial Research should investigate the development of a standard method of biological evaluation employing a film technique and that the Department of Insecticides and Fungicides, Rothamsted Experimental Station, should study various factors influencing the assessment of toxicity by biological methods and should also undertake the preparation of a pyrethrum-in-oil insecticide of known composition for use as a standard of comparison.

The programme necessitated some duplication of work, but discussions that took place from time to time between the workers concerned at the two laboratories during the progress of the investigations reduced this to a minimum.

This paper and the two following by Parkin and Green and by Martin which report the results obtained are thus closely connected and complementary.

can be stated as completely as possible and which may be repeated as desired; and (2) the working out of a biological technique, for comparing the unknown solution with this standard. This paper is mainly concerned with the second problem. The problems are largely interdependent; since biological tests are necessary to find out how alterations in various properties of the standard affect its toxicity, a laboratory-prepared standard is necessary to investigate certain aspects of the biological technique.

The results obtained fall into two categories: (*a*) those of general application to the problem of biological evaluation of toxicity, and (*b*) those having only a limited application to the problems of the biological evaluation of pyrethrum-in-oil preparations.

General survey

The following is a general survey of the work indicating the lines specially investigated.

Potter (1938) showed that, with the insecticide and technique as applied in practice, insects are killed when directly hit by particles of insecticide during application, and by contact with the film of insecticide on exposed surfaces. A complete assessment of the insecticidal values of a given preparation requires a determination of its toxicity both as a direct spray and as a film, and a direct spraying technique and a film technique were therefore devised and compared. Any such method involves the choice and standardization of test insects, a means of administering the insecticide, the standardization of the environment of the insect before, during, and after application of the insecticide, and methods of ascertaining the results and their analysis. The following is a brief résumé of the work detailed under these headings.

Insect test subjects

It must be possible to rear the test insect in large numbers in a relatively limited space under uniform conditions not too difficult to obtain or control. The species used should be as resistant as possible to disease and with the minimum of parasites and predators. It must be susceptible, but not too susceptible, to the insecticide to be tested and should not be too small or delicate to make it difficult to handle and to assess the toxic effects. The beetle, *Tribolium castaneum* Hbst., fulfilled most of these requirements and was used by us. Conditions of rearing and precautions taken to standardize the test subjects are described.

It would be better to use several species of test insects, since the estimate of relative potency obtained on a single species may not be a complete guide to its performance, but rearing large numbers of several different species under standard conditions requires much labour and apparatus, and any test that had to be repeated with several species would be time-consuming. It was urgent to obtain a practicable test rapidly and the scope of the investigation was limited to the one species.

Technique of application

(*a*) *Apparatus.* The construction and handling of the apparatus used to apply the insecticide was described by Potter (1941). It is capable of applying a wide range of dosages evenly over a circular area of 9 cm. diam., and was used in the direct spraying and the film technique.

(*b*) *Direct spraying.* In this process insects are sprayed directly with the insecticide, so that particles of fluid hit them. When pyrethrum extracts in heavy oil are used the same general principles are involved as with aqueous media, i.e. the insecticide should have physical properties sufficiently constant to give equivalent deposits for the same reservoir content throughout a series of concentrations; the distribution should be good, but this may partially be offset with non-toxic media by a large deposit which tends to neutralize small variations in amount and distribution. The substratum upon which the insects are sprayed and subsequently confined must be readily available, easily standardized, and have the right physical properties. A major difference between the technique for oil and aqueous media is that the range of deposits is much more restricted with the former, since the medium alone possesses considerable toxicity. Because of this, evenness of deposit is an important factor in the case of oil media.

(*c*) *Film technique.* In this process the substratum is sprayed and the insects subsequently confined on it. The same general principles apply to the application of a film as in direct spraying, but the importance of the factors of even distribution, and the physical properties of the substratum are, possibly, increased. In both techniques, the period for which the insects are exposed to the film is important, but in the film method the interval of time that elapses between its preparation and the confinement of the insects on it may be of considerable significance.

Possible sources of variation in direct spraying, such as the physical properties of the spray particles, their size, and the force with which they impinge on the surface of the insect, do not exist in the film technique. Every attempt was made to standardize the spraying procedure and reduce its variability to a minimum.

Factors in the technique influencing toxicity and their standardization

These factors can be grouped under the following heads: (*a*) inherent susceptibility of the insect, (*b*) environmental conditions, (*c*) period of exposure, (*d*) dosage: concentration and deposit of insecticide.

(*a*) *Inherent susceptibility of the insect.* Frequently it is possible with a given toxic agent and a given set of conditions to classify species of insects in order of inherent susceptibility, but the level of resistance of any one species does not remain constant. The

factors influencing the innate susceptibility and variation are not fully understood. Even when insects are reared from the same stock for a number of years under fairly standard conditions, and show no sign of disease or parasitism, they may still fluctuate in susceptibility from one set of cultures to the next. The insects used in these experiments were all derived from a stock bred for 4 years under conditions defined in the paper.

(*b*) *Environmental conditions.* The susceptibility of the insect and the potency of the insecticide are largely dependent upon factors external to the test subject, such as conditions of rearing and feeding, temperature and humidity, amount of handling, period of rest without food before spraying, and period of exposure of the insect to the toxic film before examination. The types of vessel in which insects are confined during and after spraying and, particularly, the nature of the substratum, are important.

The rearing conditions were standardized as far as possible, but no experimental work was done on their effect on susceptibility.

Handling of the insects during rearing and experimentation was reduced to a minimum; it is considered that handling is one of the most likely causes of uncontrolled variation. After cultures were started the insects were handled on three occasions only: when removing them into tubes before spraying, when transferring them from the tubes to the spraying dishes, and when examining them.

Preliminary experiments showed that temperature conditions during rearing and throughout the experiment have a considerable effect on toxicity. The conditions of rearing were standardized and the insects were, with very few exceptions, returned to the same conditions after treatment. The temperatures at which the insects were sprayed varied widely, since at the time there was no means available for control. The temperature immediately before spraying was also variable except for the last few experiments where the insects were pre-conditioned for about $\frac{1}{2}$ hr. before treatment. In general, little is known about the effect of humidity. It was standardized during rearing and the insects were kept after treatment at a controlled humidity.

The containers used before treatment were empty 2×1 in. tubes. The method of confinement of the insects during and after spraying underwent modification.

The substratum on which the insects are confined has an important effect on toxicity; this is marked in the direct spraying technique and is one of the most important single factors in the film technique. Much work was done on the effect of different substrata on toxicity, various fabrics and types of paper being tested as a support for the toxic film. Tricoline and a thin hardened filter paper (Whatman no. 544) largely fulfilled the requirements of both the spray and film techniques.

(*c*) *Period of exposure.* For both techniques, a decision had to be made on the period for which the insect test subjects should be allowed to remain in contact with the film of insecticide before examination. An important factor is the period during which the insect can live without food and remain unaffected by an oil similar to the one used in the insecticide preparation. The decision was to some extent bound to be arbitrary but, using *Tribolium castaneum*, about 5 days was adequate to assess the toxic effects.

(*d*) *Dosage: concentration and deposit.* Owing to variation in the susceptibility of the insects used, it is regarded unlikely, statistically, that reliable data on the relative potency of two insecticides can be obtained by subjecting the test subject to one level of dosage of each. It is possible that information to the effect that one is better or worse than the other can be elicited by such a test, but accuracy in quantitative work requires that the effect of several dosages should be employed. In the past the usual procedure was to keep the deposit constant and to vary the concentrations of a contact insecticide and ascertain its quantitative effect. A curve characteristic of a particular poison was thus obtained. A similar characteristic curve can be obtained with sprays in oil media by varying the deposits while holding the concentrations constant. Much information was obtained using both methods.

It became clear that small changes in the physical properties of the oil spray gave rise to considerable alteration in the amount of deposit per unit area for the same volumes in the reservoir: in the later experiments, therefore, deposits per unit area were checked by weighing. It was not possible to associate the variations observed with any one physical property in any simple way, although the viscosity values seemed important.

Assessments of results

(*a*) *Methods of examination and classification.* In the earlier experiments the insects were examined at laboratory temperature which was somewhat variable. This procedure was slow and uncertain, and in the later experiments the examination was made on a specially constructed constant-temperature warm plate which standardized the conditions and made the inspection easier and more rapid.

The classification of treated insects into normal, slightly affected, badly affected, moribund and dead, used over a period of years in this laboratory, was adhered to. Different schemes of classification were considered and some comparisons by different methods were made, but there appeared no sound reason for alteration.

(*b*) *Statistical treatment.* For the analysis of results involving the effect on toxicity of either concentrations or deposit, the probit-log-dosage relationship was used. The regression lines were plotted and after the determination of χ^2 for homogeneity and paral-

lelism, *M*, the log of the relative potency was calculated. Toxicity data obtained from experiments in which the concentration and deposit were successively varied led to the development by D. J. Finney, of the Rothamsted Statistical Department, of the conception of probit planes (p. 268).

Description of the technique

Test insects

The insects used were adult flour beetles (*Tribolium castaneum* Hbst.). They were reared on wholemeal flour in glass jars in a constant-temperature room at approximately 27° C. and 60–70% R.H. The flour was not standardized but was bought at a local bakery. To free it from mites and other possible infestation, it was fumigated before use with carbon disulphide at the rate of approximately 10 c.c./3200 g. of flour and 4–5 l. of space for at least 18 hr. in an air-tight glass bottle. After treatment the flour was spread out and aerated in a fume cupboard for at least 48 hr.

Round glass jars 10 cm. diam. and 21·5 cm. high were used as containers, the tops being covered with muslin. Approximately 150 g. of fumigated flour was placed in each jar, crumpled paper was put on top of the flour, and 100 adult insects were added. Two to four cultures were made up at one time.

Every attempt was made to use adults between 1 and 2 weeks after emergence, although this was sometimes not possible. There was always the risk that some older insects might be included since the original 100 insects used to start the cultures were not taken out. They were left chiefly because it was thought that more damage to the cultures would ensue from their removal than from their presence.

This technique could be further refined, but the insects produced by it proved good test subjects, and the evidence indicates that their resistance did not vary greatly during the 4 years for which they were bred. There was no indication of disease in that period. Without test insects of good quality satisfactory results cannot be obtained.

Techniques of application

Apparatus. The apparatus used for both techniques was designed by one of us and its construction and method of working were described by Potter (1941). In this series of experiments materials in an oil medium were atomized at a pressure of approx. 60 cm. mercury, those in an aqueous medium were atomized at approx. 18 cm. mercury. In the more recent experiments, the apparatus was housed in a specially designed cupboard fitted with mechanism for temperature control and with an exhaust system. The former was not used since it had not been fully tested and adjusted, but the exhaust mechanism was very useful in protecting operators and preventing contamination by the atomized insecticide, which otherwise drifted about in the room.

(*a*) *Direct spraying technique.* The insects were sprayed in Petri dishes 9 cm. diam. For the earlier experiments Tricoline circles were placed in the bottom of the dishes, but, later, circles of Whatman no. 544 filter paper were also used, so that the results remained comparable with those obtained by the film technique.

For treatment with a direct spray the insects were tipped into the dish from the 2 × 1 in. tube, placed in the machine and sprayed. They were then removed from the apparatus, and after eliminating those insects which had crawled off the circle, confined on the sprayed substratum by means of an inverted 8 cm. filter funnel. After treatment the dishes were either removed to the constant-temperature room and kept there until they were examined or dealt with as described (p. 269). In the later experiments the weight of the deposits was checked at each dilution with each solution used.

(*b*) *Film technique.* The film technique involves spraying a material of standard absorptive power evenly with a known deposit of insecticide. The test insects are confined on the sprayed surface for a given period and then examined to determine the effect.

Since the apparatus for producing the even film was available, the major difficulty with this technique was to find a substratum, constant in its physical properties, on which a lethal film could be formed with a convenient range of deposits and concentrations. Various materials were tested. Through the good offices of Mr O. B. Lean of Hawthorndale Laboratories a number of fabrics was obtained, but some of these were artificial silks which appeared to contain filling material and on this account were rejected. 'Nylon' was tried but preliminary results indicated that the slope of the regression line of probit on log deposit might be too steep for accurate quantitative evaluations to be made, and, furthermore, it was not readily available. The best fabric was Tricoline and this was and still is used for many tests.

Several paper materials were tried. Vegetable parchment and grease-proof paper were eliminated because the deposits, required to kill with those substrata, were much too small to apply and determine with any degree of accuracy, also the slope of the regression line (probit-kill against deposit) was too steep. The oil alone proved lethal at relatively low deposits. An absorbent paper, Whatman no. 50 filter paper, showed a reverse effect, but was discarded owing to the difficulty of obtaining a sufficiently high mortality with a convenient range of deposits and concentrations.

On the advice of the manufacturers of Whatman filter papers it was decided to test a thinner hardened filter paper (Whatman no. 544), and this proved satisfactory in preliminary trials. The base oil itself was not toxic when used at relatively high deposits, yet the toxicity of the spray fluid was not inhibited at weights of deposit that could conveniently be obtained.

The materials used in tests were, therefore, limited to Tricoline and hardened filter paper (Whatman no. 544). Tricoline was used for most of the earlier tests but the filter paper for the later ones, as it was somewhat more convenient in use and more readily available.

Once the substratum was decided, details of concentrations and deposits of insecticides, together with suitable exposure times to give a range of kills, had to be worked out. The time of exposure of the test insects to the film must be kept constant, and after a number of experiments it was decided that the insects should be placed on the film directly after spraying and left on it until examination; the interval finally adopted was 5 days.

For the film technique a circle of the substratum was put on a glass plate in the apparatus, sprayed to obtained the required film, and then put in the 9 cm. diam. Petri dish. The test insects were tipped from the 2 × 1 in. tube on to the sprayed surface and confined under an inverted 8 cm. filter funnel. The subsequent treatment was as in direct spraying. In the later experiments, each filter paper was weighed before and after spraying so that the exact weight of deposit was known for each replicate.

Pre-conditioning of insects. The insects were removed the afternoon before the day of spraying to empty 2 × 1 in. glass tubes, usually twenty insects per tube. The tubes were covered with muslin kept in place by a rubber band and put in a constant-temperature room at 27° C. and 60–70% R.H. Just before spraying the insects were removed from the constant-temperature room and kept in the laboratory. In the most recent experiments the insects after removal from the constant-temperature room were placed in a cool incubator at 12–15° C. for ½ hr. before spraying commenced. This was done because the laboratory temperature varied greatly throughout the year, and the insects are easier to handle at this low temperature.

Assessment of results

Method of examination

In the earlier experiments the inspection was made at laboratory temperatures, but later, a thermostatically controlled warm plate specially constructed by one of us (C. P.) was used. This plate, with a surface of 34 × 27·5 cm., when tested by a thermocouple was found to maintain a surface temperature of between 35 and 40° C.; the central area of 602 sq. cm. was 37·5–40° C. except for a very small portion above the thermoregulator. The warm plate greatly facilitated inspection, and much more standard results were obtained by its use.

During the inspection the insects were examined individually with the naked eye or under a lens or low-power binocular. They were placed in five categories: (*N*) unaffected, capable of free movement; (*S*) slightly affected—suffering some inhibition of movement; (*B*) badly affected with definite signs of paralysis and powers of movement gravely restricted or impossible; (*M*) moribund, in which the insects were only capable of twitching antennae or legs; and (*D*) apparently dead, no movement being observable.

Statistical analysis

Usually the classes *B*, *M* and *D* were taken together and their percentage values converted to probits, plotted against log concentrations or deposits. The usual statistical technique, using the principle of *maximum likelihood*, was employed for determining $M = \log \frac{\text{potency of test preparation}}{\text{potency of standard}}$. The antilog of *M* gave the relative arithmetical values for the potencies. In one case (p. 274) marks were awarded to the categories, but little of value was obtained by this procedure.

It frequently happened, particularly with the oil sprays, that the results plotted in this way showed a break in the line above and below probits of 6 and 4 respectively. These figures are only approximate, but it is very doubtful whether values above and below them can be obtained accurately with the numbers of insects conveniently used in trials of this kind.

The above method is usual where one independent variable, such as concentration or deposit, is related to percentage effect. In some experiments two independent variables were concerned, as both concentration and deposit were altered successively. Mr D. J. Finney (1943) of the Rothamsted Statistical Department suggested that, as an extension of the usual findings in the analysis of toxicity tests by means of probit regression lines, the probit of the kills might be related linearly both to the logarithm of the concentration and to the logarithm of the deposit. If this be so, the points determined by the log concentration, log deposit, and probit kill should lie on a plane for each technique (direct spray or film), and he therefore developed the necessary statistical technique for the fitting of such planes to the data. It is the subject of a separate publication by him, but the main facts are outlined later. The employment of this mode of analysis demands the replacement of the use of *M* by the concept Δ, defined as the difference (in probit units) between the kills obtained as represented by two parallel probit planes.

Examination of some physical factors affecting the technique

Relationship between volume in reservoir and deposit

There would be a great simplification in technique and reduction in the time required for carrying out a programme of spraying, if the amount placed in the reservoir could be taken as a relative measure of the amount of deposit formed on the surface, but it is clear that for oil solutions having different physical

properties this would probably not be true. It was thus essential to investigate for oil sprays the relationship between the total volume of liquid sprayed at constant pressure and the amount distributing itself over the surface in the form of a film, and to ascertain how sensitive to change is this relationship. Time only permitted trials with oil sprays corresponding in type to one of the commercial preparations and with some of the standards.

In the earlier experiments it was assumed that the deposit per unit area could be ascertained by preliminary trials carried out with the base oil. If this were not strictly true the results in these cases would not be invalidated, since comparisons were then being made between equivalent concentrations prepared from the same stock solutions, and the main object was to ascertain the most suitable surface for use. In tests in which a commercial preparation was compared with a standard not equivalent in make, it was imperative to learn whether the composition of the spray fluid effected the weight of deposit. Numerous tests were made to correlate the volume of fluid in the reservoir with the amount of deposit, and tared glass dishes of known area, glass disks, circles of Tricoline, and filter paper were used for determining the deposit. When the data were plotted (deposit mg./cm.^{2} *v.* ml. in reservoir) the points fell near to, but not exactly on, a straight line. In some cases, owing probably to viscosity effects and the shape of the reservoir, the line was slightly parabolic. In the earlier experiments a Petri dish was used for receiving the deposit, but since the walls might have an effect both on its distribution and weight, the two methods of receiving the deposit—disk or dish—were compared in a series of experiments (25 Sept. 1941), the tests for each volume of Wakefield half-white oil (specification on p. 266) in reservoir being replicated five times (see Fig. 1).

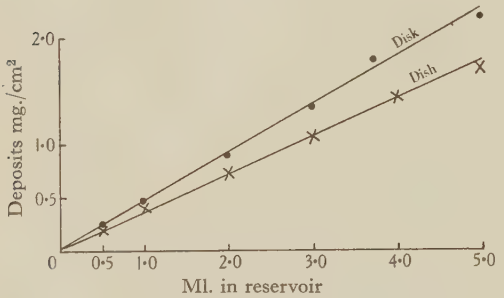

Fig. 1. Relation between reservoir content and deposit. Deposit on disk and dish. The figure shows the effect of the walls of a Petri dish upon the deposits of a heavy petroleum oil contrasted with those on a flat disk of the same area.

The walls of the dish have a shielding effect and a consistently higher deposit is given on the disk, the mean ratio being 0·798. A disk was therefore used in later work. Weighed circles of the fabric or paper surface were superimposed upon it, the film sprayed on to them and reweighed. This is recommended for use as a routine, unless a dish is to be used for holding the insects as in the direct-spraying technique, when the circles are sprayed in a dish.

The effect of the nature of the spray fluid on weight of deposit

Two standard preparations of the same pyrethrin I content, one with the natural colour of a pyrethrum extract (sp. gr. = 0·84) and the other rendered colourless (sp. gr. = 0·867), were compared with each other and with the Wakefield base oil (half-white) (sp. gr. at 15·5° C. = 0·880). Tared circles of Tricoline of a mean area 63 cm.^{2} were used, and the weighings carried out in triplicate on disks (see Fig. 2).

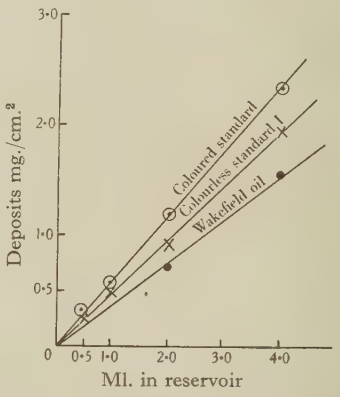

Fig. 2. Relation between reservoir content and deposit. Deposits from different pyrethrum-in-oil preparations.

The nature of the spray affects the relationship between the volumes sprayed and the weight of the resulting deposit, the differences being of such a magnitude as not to be negligible in toxicity work.

The experiment was repeated, circles of hardened filter-paper (Whatman no. 544) being used instead of Tricoline (see Table 1).

TABLE 1. *Effect of nature of spray fluid on weight of deposit*

Ml. in reservoir	Coloured standard pyrethrum-in-oil mg./cm.^{2}	Colourless standard no. 1 pyrethrum-in-oil mg./cm.^{2}	Wakefield half-white oil mg./cm.^{2}
0·25	0·176	0·168	—
0·5	(1) 0·293 (2) 0·338	0·249	—
1·0	0·600	0·469	—
2·0	(1) 1·242 (2) 1·148	0·922	0·694
4·0	2·486	1·802	1·431
6·0	—	2·633	—

Relationship between weight of deposit and apparent viscosity

No simple relationship was found between the physical properties of the oil sprays and the deposit, but an indication that in certain cases there is a rough correlation between apparent viscosity values (as measured by times of flow at various pressures) and the deposits, is shown by the results obtained when two colourless pyrethrum-in-oil standards were compared with the oil alone and a commercial preparation. The deposits were determined in triplicate in the usual way using tared circles of filter paper (9 cm. diam.) and a gauge pressure of 60 cm. mercury. The mean values are shown in Fig. 3.

The relative rates of flow were determined in a standard no. 2 U-tube viscometer immersed in a thermostat at approximately 23·5° C. Both ends were attached through a three-way tap and pressure

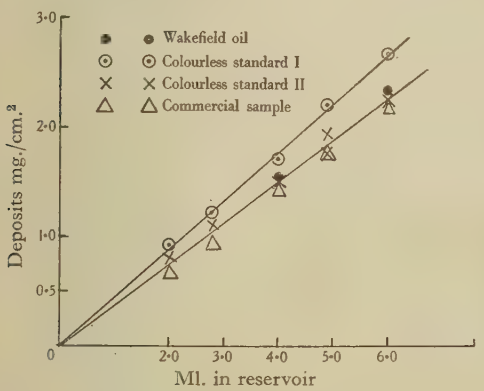

Fig. 3. Relationship between reservoir content and deposit. Deposits for various pyrethrum-in-oil preparations. Tests carried out to show relationship between apparent viscosities and deposit. To be compared with Fig. 4.

gauge with a water pump, which could be used alternatively for drawing the pyrethrum-oil solution above the top mark or for exerting a partial vacuum pull in the reverse direction. The pressure gauge contained paraffin oil of 0·864 sp. gr. and was fitted with a capillary leaking device of the flowmeter type; the water pump could be set to give a pressure (vacuum) of approximately a given value. It was not perfectly constant and the apparatus could be improved for accurate work; the gauge, however, was read about every 10 sec. and the mean value taken. The time for each fluid to run between the two marks was determined by stop-watch. In Fig. 4 the mean pressures are plotted against the time of flow.

The results tend to show an inverse relationship between the time of flow at 23·5° C. at different pressures and the weights of the deposits in the spraying machine. Since such factors as adhesion, cohesion, and turbulence of flow may all be involved, it does not seem probable that by adjusting the physical constants of the spray oils to certain values, any uniform correlation between deposit and reservoir content for different preparations could be secured.

Effect of sludge on certain physical properties in the pyrethrum-in-oil sprays

One exceptional sample of a commercial preparation contained an amount of sludge-like material which could be centrifuged out; it gave an opportunity for determining the effect of this oil-insoluble semi-solid matter on the weights of deposit and upon toxicity data (p. 271). The presence of much sludge in a pyrethrum-oil spray may lead to complete blocking of the nozzle, or at least to a progressive change in the weight of the deposit, owing to choking of the tubes and orifices in the atomizer.

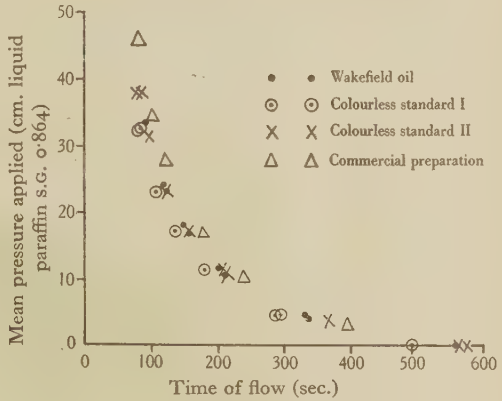

Fig. 4. Relationship between reservoir content and deposit. Apparent viscosities of various oils at different pressures. A comparison of this figure with Fig. 3 shows the relationship between apparent viscosity and deposit of various oil preparations.

The effect was ascertained of centrifuging a commercial pyrethrum-in-oil preparation upon the pyrethrin I content, upon the weight of deposit for several reservoir contents, and upon the insecticidal value. The commercial sample containing the sludge was analysed before and after centrifuging by Dr J. T. Martin: the pyrethrin I contents did not differ significantly. There was no estimable loss of pyrethrin I through centrifuging, but Dr Martin reported that the analysis was rendered materially easier, since the tendency to emulsification during the process of analysis was markedly less in the case of the centrifuged material. Both samples were diluted with Wakefield oil to a content of 0·8% pyrethrin I and used in determining the weight of deposit and in insecticidal tests. The colourless standard I (p. 273) and the Wakefield half-white oil were tested at the same time.

In descending order of magnitude the weights of

deposits per unit area for equivalent reservoir contents were: colourless standard > commercial preparation (not centrifuged) > commercial preparation (centrifuged) > base oil. The difference in the weights of deposit between the centrifuged and non-centrifuged commercial samples was small. Experiments on the effect of sludge on toxicity are dealt with later (p. 271).

Effect of deposit differences on insecticidal values

Table 1 shows that the weights of deposits for the same amount of liquids in the reservoir are much greater with the coloured than with the colourless standard pyrethrum-in-oil preparation, 4 c.c. of the former giving a deposit per unit area of about the same weight as 6 c.c. of the latter. Thus, very misleading results might ensue if the reservoir content were accepted as equivalent to the dosage for all samples. Certain insecticidal data were accumulated for these two standards (Table 12, p. 276). The results in which the probits are plotted against log deposit (*B*) and against log volume in the reservoir (*A*) are given in Fig. 10. Both *A* and *B* show the coloured standard to be more toxic than the colourless, but in *B* the coloured standard is about × 1·28 as potent as the colourless, whereas in *A* it is of the order of × 1·7. The difference in the activity of the two standards is discussed later, but the chemical analysis indicates the lower of these two values to be more nearly correct.

It is thus clear that the reservoir content cannot be taken as a measure of either deposit or dosage in pyrethrum-in-oil sprays, and the deposits should be weighed for each test.

COMPARISON BETWEEN DIRECT SPRAY AND FILM TECHNIQUE

The first series of tests (20 May 1941) was carried out with a textile fabric substratum using all combinations of three deposits (0·29, 0·57, and 1·08 mg./cm.^{2}) with four concentrations (50, 100, 200 and 400 mg./100 ml.) expressed in terms of pyrethrin I, and Wakefield half-white oil* as medium. The deposits were determined by spraying known volumes of Wakefield half-white oil on to a tared disk of known area, which was afterwards weighed. It was not realized at the time that the introduction of pyrethrum extract might alter the weight of deposit. In the present case any small error so introduced was the same for both techniques and did not invalidate the results obtained. The disk was covered with a surface of a loosely woven fabric (an inferior fabric of the Tricoline type).

In the second series (19 June 1941) a similar fabric was used but with a wider range of deposits (0·4, 0·11, 0·28, and 0·74 mg./cm.^{2}), and four concentrations of pyrethrin I (100, 200, 400 and 800 mg./100 c.c.). The data are shown in Tables 2 and 3 and those of the first series in Fig. 5. In both series

TABLE 2. *Comparison between direct spray and film technique. Experiments 20 May 1941*

Insecticide: pyrethrum in heavy oil (Wakefield half-white).
Substratum: fabric. Test subject: adult *Tribolium castaneum* Hbst.

Deposit mg./cm.^{2} ...	0	0·29	0·57	1·08
Pyrethrin I conc. mg./100 ml.				
		Direct spray % (*B*, *M* and *D*)		
0	0* (0/22)	3·3† (1/30)	0† (0/27)	0† (0/29)
50	7·1* (2/28)	0 (1/27)	10·0 (4/29)	16·5 (6/30)
100	0* (0/28)	49·6 (15/29)	64·0 (19/29)	60·9 (15/24)
200	10·7* (3/28)	89·6 (27/30)	96·1 (26/27)	100 (31/31)
400	3·2* (1/31)	100 (28/28)	100 (30/30)	100 (19/19)
	Av. in unsprayed control 4·2 %			
		Film technique % (*B*, *M* and *D*)		
0	—	6·9† (2/29)	3·4† (1/29)	6·7† (2/30)
50	—	6·4 (3/29)	11·06 (4/27)	25·5 (8/28)
100	—	30·4 (10/30)	47·8 (14/28)	59·0 (17/28)
200	—	82·0 (24/29)	96·2 (27/28)	92·6 (26/28)
400	—	100 (29/29)	100 (29/29)	100 (17/17)

Threefold replication—10 insects at a time. Figures are percentages allowing for a control figure of 4·2 %. Figures in brackets are actual proportions obtained. *B* = badly affected; *M* = moribund; *D* = apparently dead.

* Unsprayed controls. † Base oil alone—controls.

* This oil had the following characteristics: 10 % distilled at 298–319° C., 80 % at 319–388° C.; flash point (closed) = 310° F.; viscosity Redwood 1 at 70° F. = 104 (33·2 centistokes at 20° C.); sp. gr. at 15·5° C. = 0·880; unsulphonatable residue 88 % by volume.

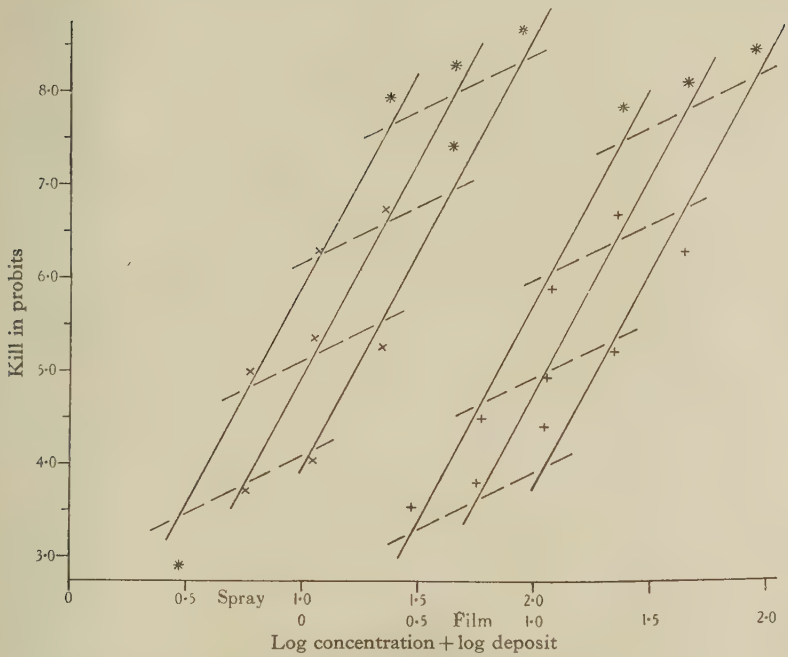

Fig. 5. Toxicity of pyrethrin oil spray to *T. castaneum*. × direct spray technique; + film technique; * 0% or 100% kill; —— effect of change in concentration for fixed deposit; – – – effect of change in deposit for fixed concentration.

TABLE 3. *Comparison between direct spray and film technique. Experiments* 19 *June* 1941

Insecticide: pyrethrum in heavy oil (Wakefield half-white).
Substratum: fabric. Test subject: adult *Tribolium castaneum* Hbst.

Deposit mg./cm.^{2} ...	0		0·04		0·11		0·28		0·74	
Pyrethrin I conc. mg./100 ml.										
				Direct spray % (*B*, *M* and *D*)						
0	0	(0/30)	7·1*	(2/28)	0*	(0/29)	0*	(0/19†)	7·7*	(2/26)
100	—		1·8	(1/23)	10·1	(3/24)	19·3	(6/28)	92·4	(25/27)
200	—		10·1	(3/24)	52·6	(14/26)	58·2	(16/27)	96·5	(28/29)
400	—		75·5	(16/21)	85·3	(24/28)	100	(28/28)	100	(30/30)
800	—		100	(31/31)	100	(28/28)	100	(29/29)	100	(30/30)
				Film technique % (*B*, *M* and *D*)						
0	—		0*	(0/27)	3·2*	(0/31)	3·4*	(1/29)	0*	(0/28)
100	—		0·6	(1/31)	1·1	(1/27)	23·0	(8/32)	89·0	(25/28)
200	—		4·1	(2/30)	19·3	(6/28)	48·6	(14/28)	100	(30/30)
400	—		69·2	(21/30)	100	(30/30)	100	(31/31)	96·2	(26/27)
800	—		74·3	(21/28)	100	(30/30)	100	(35/35)	100	(28/28)

Threefold replication—10 insects at a time. The figures are percentages allowing for a control figure 2·63%. Figures in brackets are the actual proportions obtained. *B* = badly affected; *M* = moribund; *D* = apparently dead.

* Base oil alone—controls.

† In one batch all escaped but 1, which was dead; it was discarded.

tests were also made of the effect of the base oil (without pyrethrins) at the several deposit rates.

Mr Finney* examined the data and reported his conclusions as follows:

'In neither experiment was there any indication of a toxic effect of the base oil alone, though it is, of course, possible that deaths might occur at higher deposits.

'Probit planes were fitted to the probits (after adjustment for the mortality among the controls) relating them to the log concentration and log deposit. In the second experiment there was considerable heterogeneity of the points about the planes, but this did not appear to be the result of any systematic departure from the hypothesis of a plane relationship. There was, for example, no evidence of an interaction between the two dosage factors such as would cause a given alteration in deposit to have an effect on kill which varied with the concentration used; the two factors appeared to be entirely independent in their effects.

'It was thought that the kill might be dependent only on the total amount of pyrethrin deposited (obtainable as the product of concentration and deposit), but this hypothesis was clearly disproved as increases in concentration had much greater effect on the kill than the corresponding increases in deposit. Thus in the first experiment a doubling of the concentration increased the kill to the same extent as did multiplication of the deposit by 13·5; in the second experiment the difference was much less, but a doubling of concentration was as effective as a multiplication of deposit by 3·4. A further test with Tricoline (p. 271) confirmed these results, but one experiment using hardened filter paper (Whatman no. 544) indicated that with this substratum the position was reversed.

'The film technique gave slightly lower kills than the direct spray for the same dosages (here by the term "dosage" is to be understood the combination of a concentration and a deposit), but in neither experiment was this difference significant. When only concentration is being tested, it is usual to express differences in potency by M, the difference in the log concentrations which give the same kill, or by the antilogarithm of M, which measures the ratio of equally toxic concentrations. If the probit lines are parallel the same value is obtained at whatever level of kill the comparison is made. This measure cannot, however, be generalized to the present analysis and instead it is suggested that the difference between the two techniques (spray and film) can best be measured by the mean probit difference, Δ, which is the difference (in probit units) between the kills obtained by the two techniques at any given dosage. In each of these experiments the probit planes for the two techniques were parallel, and Δ therefore has the same value for every selected dosage. In the first experiment $\Delta = 0{\cdot}169 \pm 0{\cdot}136$, and in the second $\Delta = 0{\cdot}235 \pm 0{\cdot}197$. Neither of these values alone can be considered significant, but the mean value, $0{\cdot}190 \pm 0{\cdot}119$, exceeds its standard error to an extent which could occur by chance in only 10% of cases and suggests that there may be a real difference. The meaning of a mean probit difference of this amount is illustrated in Table 4 which shows the percentage kill expected by the film method for a series of values by the direct spray.

TABLE 4. *Relative percentage kills by spray and film technique*

Spray	25·0	50·0	75·0	90·0	95·0	99·0	99·90
Film	19·3	42·5	68·6	86·2	92·7	98·4	99·81

'The two experiments gave results in fairly good agreement over the range of concentrations and deposits common to them. Table 5 shows the kills predicted from the probit planes in the two experiments.

TABLE 5. *Comparison of predicted percentage kill in experiments I and II*

Concentrations (mg./100 ml.)	100		200	
Deposit (mg./sq. cm.)	0·3	0·6	0·3	0·6
Spray I	44·2	59·1	89·7	94·9
Spray II	38·1	64·3	81·2	94·0
Film I	37·7	52·4	86·3	92·9
Film II	29·6	55·3	74·2	90·6

'These figures again emphasise the greater effectiveness of an increase in the concentration of pyrethrin in the oil, than of an equivalent increase in the deposit of a given concentration.'

The film technique is thus less effective than the direct spraying of the insects, and this is shown in both the cases (1) in which deposit is kept constant and the concentrations varied, and (2) in which deposit is varied for constant concentration. Finney also pointed out that M (the relative potencies of the pyrethrum-in-oil as determined by the two methods) is expressed in logs by Δ/b, where Δ is the mean probit difference and b the regression coefficient on the concentration or on the deposit, according as one or the other is held constant. The standard error of M is *approximately* that of Δ divided by b.

The relative potencies in logs for constant deposit and varied concentration:

Test I $M = 0{\cdot}036 \pm 0{\cdot}029$
Test II $M = 0{\cdot}060 \pm 0{\cdot}050$

The relative potencies in logs for constant concentration and varied deposit:

Test I $M = 0{\cdot}136 \pm 0{\cdot}118$
Test II $M = 0{\cdot}106 \pm 0{\cdot}089$

* Mr Finney (1943) prepared for publication a paper in which his detailed analysis of the first of the above series is embodied. Fig. 5 is taken from his paper by kind permission of the Editors of *The Annals of Applied Biology*.

Thus for constant deposit and varied concentration, the tests show the direct-spray technique to be from × 1·085–1·15 as effective as the film technique, and for constant concentration and varied deposit × 1·28–1·37 as effective.

In the first series of the above tests where the data were homogeneous there was no evidence of a significant difference in homogeneity between the direct spray and film technique. In the second series in which some heterogeneity occurred in both sets of results, the data from the film technique were slightly more heterogeneous, but there is nothing to show that one technique can be regarded as significantly better than the other in this respect.

THE APPLICATION OF THE TECHNIQUES

The direct-spray technique

The determination of the degree of loss of activity of the pyrethrins

Two series of tests were carried out by direct spraying, *Tribolium castaneum* Hbst. being used as test subject. Usually in this technique the deposit is purposely made large so that fluctuations of a minor kind may be negligible in their effect. After spraying, those insects which had moved under or off the circle of fabric were eliminated. The sprayed insects were generally covered by an upturned funnel, but a few by butter muslin. In these experiments the insects after treatment were kept in a cellar maintained at a relatively constant temperature of 18–20° C. for 2 days and then removed to the constant-temperature room (temp. 27° C., R.H. 60–70%) for a further 2 days, after which they were examined by the method outlined above (p. 263).

Series I (16 *July* 1942). *Substratum: Tricoline. Material used: a gum-tragacanth-pyrethrum concentrate dispersed in water.* The purpose of the experiments was to ascertain whether the chemical and biological tests would agree in detecting and estimating a loss of activity induced by an exposure of 14 days to a temperature of 50° C. Preliminary trials were made in which the pyrethrin content was reduced by 10% by dilution. In this process the viscosities of the solutions of the diluted material were different from those of the original material, and this led to an alteration in the slope of its regression line (probits *v.* concentration). The relative potencies were of the same order as the ratio of the pyrethrins in the two samples, and it was clear that a loss of 10% could be detected, but that for reliability it would be necessary to use solutions possessing the same physical properties throughout the tests.

The concentrate (total pyrethrin content 0·5% w/v) contained in a bottle filled nearly to the brim and closed with a screw cap, was exposed to a temperature of 48–50° C. in a temperature-regulated oven. After 14 days its insecticidal activity was compared with an exactly similar sample which had been stored in a refrigerator. This was carried out by preparing from each sample dilutions of apparently the same total pyrethrin content, by means of a solution in 0·1% sulphonated lorol of the gum, prepared in exactly the same way as the basis of the pyrethrum preparations. The proportions of wetter and gum were kept constant for all the dilutions throughout the tests. For all dilutions 5 c.c. were used in the reservoir and spraying was done at a pressure of 18 cm. (mercury). The deposit was determined for each concentration and was of the order of 10 mg./cm.² (see Table 6 and Fig. 6).

A chemical analysis* carried out by Drs Martin and Harper showed a difference in pyrethrin content of 11·8% thus:

	Pyrethrin I %	Pyrethrin II %	Total pyrethrins %
Sample kept at 0° C.	0·24	0·27	0·51
Sample kept at 50° C. for 14 days	0·21	0·24	0·45

I.e. that the sample kept in the refrigerator is × 1·13 as rich in pyrethrins as the sample exposed to a temperature of 50° C. for 14 days. By computation from the data the biological tests showed a difference of 12·7%, i.e. the sample stored in the cold was × 1·15 as toxic as the one kept at 50° C.

Series 2 (29 *July* 1942). *Substratum: filter paper Whatman no.* 544. *Material used: a pyrethrum-paraffin concentrate.* Samples of the concentrate, containing 0·64% total pyrethrins, were exposed for 14 days to temperatures of 0 and 50–58° C. The purpose was as in series 1. The dilutions at which the preparations were tested were made by dissolving the concentrates in benzene and diluting with a benzene solution of the paraffin used as a base, so that its content remained the same throughout the tests and the viscosities of the several solutions were approximately the same. There was some difficulty in carrying out the tests owing to clogging of the nozzle and the weights of the deposits for similar volumes were rather variable; the tests were therefore not so accurate as in series I. In the sample kept in the refrigerator (A) the deposits ranged from 0·8 to 1·0 mg./cm.², and with the sample exposed to 50–58° C. three concentrations gave deposits from 0·85 to 1·05 mg./cm.², the atomizing pressure was approx. 29 cm. of mercury. The deposit for the lowest concentration in the latter was about 0·6 mg. but little weight was attached to the result and it could have been discarded (see Table 7 and Fig. 7).

A statistical examination of the data given in Table 7 showed that the sample kept in the refrigerator was × 1·2 as toxic as the sample exposed to 50–58° C. or that there had been a loss of activity of about 16·5%. A slightly less drastic exposure

* The pyrethrin I content was determined by a modified Wilcoxon-Holaday method and pyrethrin II by the methoxyl method.

TABLE 6. *Direct-spray technique*

Comparative toxicities of an aqueous pyrethrum preparation exposed to temperatures of 0 and 50° C. for 14 days (16 July 1942).

Substratum: Tricoline. Test subject: adult *Tribolium castaneum* Hbst.

Mean conc. mg./100 ml. total pyrethrins	Mean log conc.	$B+M+D$ %	Experimental probit	No. of insects	Notes
Exposed to 0° C.: A					
16	1·2041	75·6	5·6935	90	
12·8	1·1072	58·8	5·1459	86	A. χ^2 for $n'-2=2=0{\cdot}062$. Data not heterogeneous.
10·2	1·0086	39·4	4·7311	94	
8·2	0·9138	24·4	4·3065	86	A/B $\chi_b^2=0{\cdot}194$. Lines do not depart significantly from parallelism.
Exposed to 50° C.: B					A/B $M=\log\frac{\text{potency of A}}{\text{potency of B}}=0{\cdot}0592\pm0{\cdot}022$.
16*	1·2041	63·0	5·3319	92	
12·8*	1·1072	46·7	4·9172	92	A/B A is 1·146 times as toxic as B.
10·2*	1·0086	29·9	4·4727	87	B. χ^2 for $n'-2=2=0{\cdot}175$. Data not heterogeneous.
8·2*	0·9138	18·0	4·0846	89	

The concentrations are based upon the assumption that the original preparation contained 0·5 % w/v total pyrethrins.

* These are apparent values, the induced loss not being accounted for. The mean deposits varied between 9·25 and 10 mg./cm.^{2}

Only 1 % of the insects were dead in the controls. B = badly affected; M = moribund; D = apparently dead; based upon five replicates of approximately twenty insects each.

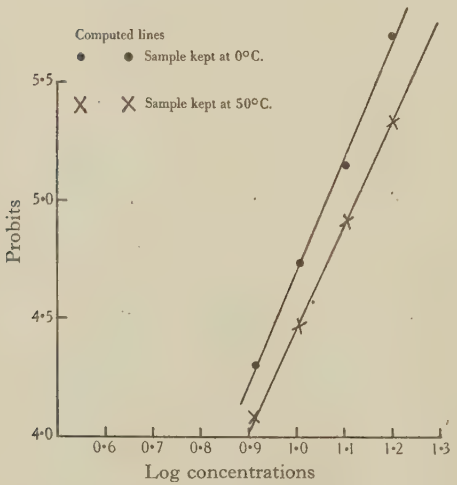

Fig. 6. Direct spray technique to estimate loss of toxicity of pyrethrins in an aqueous medium due to exposure to 50° C. for 14 days (16/7/42).

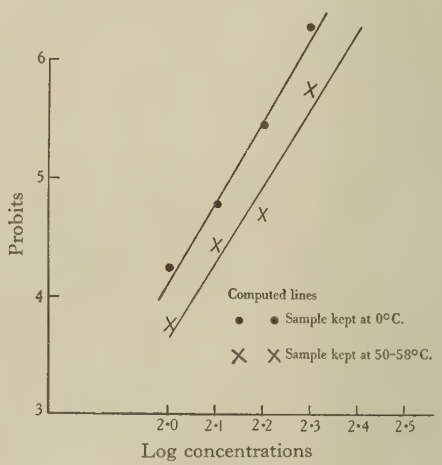

Fig. 7. Direct spray technique to estimate loss of toxicity of pyrethrins in a paraffin medium due to exposure to 50–58° C. for 14 days.

TABLE 7. *Direct-spray technique*

Comparative toxicities of a paraffin-pyrethrum preparation exposed to temperatures of 0 and 50–58° C. for 14 days (29 July 1942).

Substratum: Whatman filter paper no. 544. Test subject: adult *Tribolium castaneum* Hbst.

Mean conc. mg./100 ml. total pyrethrins	Mean log conc.	$B+M+D$ %	Experimental probit	No. of insects	Notes
Exposed to 0° C.: A					
200	2·301	90·0	6·282	90	
160	2·204	67·3	5·449	98	A. $\chi^2=0{\cdot}848$ for $n'-2=2$. Data not heterogeneous.
128	2·107	41·2	4·779	96	
102·5	2·011	22·1	4·231	95	
Exposed to 50–58° C.: B					
200*	2·301	77·3	5·747	88	
160*	2·204	38·5	4·709	96	
128*	2·107	28·4	4·429	95	
102·5*	2·011	10·7	3·760	93	B. $\chi^2=5{\cdot}366$ for $n'-2=2$. Data not heterogeneous.

A/B: $\chi_b^2=0{\cdot}5446$. Lines do not depart significantly from parallelism. $M=\log\frac{\text{potency of A}}{\text{potency of B}}=0{\cdot}079$. A is 1·2 times as toxic as B.

The concentrations are based upon the assumption that the original preparation contained 1·0% total pyrethrins.

* These are apparent concentrations, the induced loss not being accounted for.

The mean deposit series A = 0·8–1·0 mg./cm.², series B first three tests 0·85–1·05 mg./cm.²

There were no deaths in the control. B = badly affected; M = moribund; D = apparently dead; based upon five replicates of approximately twenty insects each.

(48–50° C. for 14 days) was employed in the samples used for the chemical determination of the pyrethrins; the results obtained were:

	Pyrethrin I %	Pyrethrin II %	Total pyrethrins %
Exposure to 0° C.	0·28	0·36	0·64
Exposure to 48–50° C.	0·24	0·32	0·56

I.e. the sample exposed to 0° C. is ×1·14 as rich in pyrethrins as the sample exposed to 50° C. or a difference of 12·5%. On the whole the agreement can be regarded as good. These tests indicated that a loss as small as 10% could be detected by the technique: no attempt was made to distinguish smaller differences.

Film technique

(*a*) *Influence of substrate on the relative effectiveness of increases in concentration and deposit*

After the spray and film technique had been compared using a fabric substratum it was desirable to obtain data for the film technique using Whatman filter paper no. 544 as substratum in order to discover (1) a suitable range of concentrations and deposits for use with this material, and (2) its effect on the relative toxicity caused by increases of concentration and increases in deposit. The following are the details of the experiment:

Date 17 *Sept.* 1941. Substratum: hardened filter paper Whatman no. 544. Insecticide: commercial extract of pyrethrins in heavy oil. Medium: Wakefield half-white oil.

Tests were made at seven weights of deposit and at four concentrations of pyrethrins. Despite discrepant points Mr Finney's analysis showed that the data were satisfactorily fitted by a plane (see Table 8).

In contrast with the earlier experiments using a textile fabric, increasing the deposit had a greater effect on the kill than increasing the concentration. Doubling the concentration was only as effective as ×1·54 the deposit. These results need confirmation.

Date 22 *Nov.* 1941. Substratum: Tricoline. Insecticide: standard solution I (p. 273). Genuine Tricoline being available a further experiment was carried out at two concentrations and five deposits. The eight points obtained were remarkably close to the probit plane. The effect of increases of concentration on percentage kill was relatively greater than the effect of increases in deposit, a doubling of the concentration being equivalent to ×6·8 the deposit. This is in agreement with earlier experiments (see Table 9).

(*b*) *Effect of presence of sludge on toxicity*

It was demonstrated in earlier experiments that the toxic effect of a spray fluid may depend upon the surface upon which the insects are placed. If the surface is altered by the spray a new and complicating factor is introduced. The presence of much sludge or slime may modify a surface, and the complication due to its presence, other than the mere mechanical one of blocking the nozzle, must be considered. Three sets of biological trials were carried out to test the effect of sludge on toxicity: (1) 22 Nov. 1941. Tricoline was used as a substratum. A commercial preparation containing much sludge, the same preparation with the sludge centrifuged out and a colourless pyrethrum standard, for

purposes of comparison, were tested. Dilutions were made with Wakefield half-white oil, two concentrations and five deposits were employed for each preparation. The insecticidal data for the standard lay remarkably close to the probit plane, but there was much irregularity with both the centrifuged and uncentrifuged commercial preparation, this being particularly true of the latter. No other deductions could be drawn. In this case, however, the weights of each deposit were not determined. (2) 11 Dec. 1941. Tricoline was used as the substratum in tests which compared the uncentrifuged commercial sample with the colourless standard no. 1, both diluted with Wakefield oil. The concentrations of pyrethrin I were respectively 0·7 and 0·8%, and the weights of all the deposits were determined. Omitting one very aberrant test of the standard, the data for the standard and the commercial product show a good degree of homogeneity, χ^2 for the standard being 0·304 for $n'-2=1$ and for the commercial product 0·607 for $n'-2=2$. The departure from parallelism is not significant ($\chi_b^2=0{\cdot}906$), but there is possibly a slight break at the bottom of the regression line for the uncentrifuged material which causes it to cross the line for the standard. The relative potency, commercial product to standard, was 0·89 in close agreement with the ratio of contents of pyrethrin I = 0·875 (see Table 10 and Fig. 8).

The commercial sample (a concentrate) was probably highly exceptional, in that the amount of sludge was great, but Exp. 2 indicates that useful data can be obtained with such samples and that sludge has no significant effect upon toxicity. (3) 25 Feb. 1942. Whatman filter paper no. 544 was used as sub-

TABLE 8. *Investigation of film technique*

Insecticide: pyrethrum in heavy oil (Wakefield).
Substratum: Whatman filter paper no. 544. Test subject: adult *Tribolium castaneum* Hbst. (17 Sept. 1941).

Deposit mg./cm.² ...	0	0·320	0·472	0·697
Conc.	$B+M+D$	$B+M+D$	$B+M+D$	$B+M+D$
% pyr. I w/v	%	%	%	%
0	0 (0/38)	0* (0/44)	2·44* (1/41)	0* (0/38)
0·1	—	—	—	—
0·2	—	—	—	—
0·4	—	—	—	29·8 (12/39)
0·8	—	9·6 (6/55)	28·6 (8/27)	26·95 (7/25)

Deposit mg./cm.² ...	1·072	1·524	2·479	3·891
Conc.	$B+M+D$	$B+M+D$	$B+M+D$	$B+M+D$
% pyr. I w/v	%	%	%	%
0	0* (0/40)	2·86* (1/35)	4·44* (2/45)	61·76* (21/34)
0·1	8·7 (4/40)	1·2 (1/38)	50·6 (20/39)	100 (40/40)
0·2	27·6 (10/35)	32·3 (13/39)	80·3 (29/36)	100 (40/40)
0·4	49·3 (18/36)	65·4 (27/41)	89·85 (36/40)	—
0·8	67·2 (23/34)	88·2 (38/43)	—	—

Figures are percentages corrected for a control figure of 1·4%.
Figures in brackets are actual proportions obtained.
B = badly affected; M = moribund; D = apparently dead; based on two replicates of approximately twenty insects each.
* Controls sprayed with oil alone.

TABLE 9. *Investigation of film technique*

Insecticide: pyrethrum in heavy oil standard solution I.
Substratum: Tricoline. Test subject: adult *Tribolium castaneum* Hbst. (22 Nov. 1941).

Deposit mg./cm.² ...	0	0·19	0·38	0·77	1·54	2·31
Conc.	$B+M+D$	$B+M+D$	$B+M+D$	$B+M+D$	$B+M+D$	$B+M+D$
% w/v	%	%	%	%	%	%
0	1·72 (1/58)	—	—	—	1·81 (1/55)	1·75 (1/57)
0·467	—	—	11·5 (8/61)	20·8 (12/54)	36·1 (22/59)	41·9 (22/49)
0·8	—	22·2 (13/55)	35·9 (23/62)	54·7 (30/54)	71·9 (42/58)	—

Percentages corrected for a control of 1·8%.
Figures in brackets are actual proportions obtained, $B+M+D$: number of insects used in test.
B = badly affected; M = moribund; D = apparently dead; based on three replicates each of approximately twenty insects.

stratum. For both the standard and the centrifuged commercial product χ^2 was relatively high (respectively 6·8 and 5·1 for $n-2=2$); the relative potencies could be determined, but the uncentrifuged experiments gave no definite evidence that sludge affects toxicity to any great degree, the effect of its presence needs fuller study and the advisability of centrifuging it out should be ascertained.

TABLE 10. *Film technique to compare the toxicities of pyrethrum-in-oil preparations*

Comparison between colourless standard I and a commercial sample.
Substratum: Tricoline. Test subject: adult *Tribolium castaneum* Hbst. (11 Dec. 1941).

Deposit mg./cm.²	log (deposit × 10)	$B+M+D$* %	Experimental probits	No. of insects	Value of $M=\log\frac{\text{potency of B}}{\text{potency of A}}$
A. Colourless standard I (pyr. I=0·8 % w/v):					
0·187	0·272	25·9	4·354	56	
0·399	0·601	32·9	4·557†	66	
0·785	0·895	79·3	5·817	60	$M=\bar{1}\cdot9511$
1·583	1·200	96·4	6·799	57	Antilog $M=0\cdot893$
B. Commercial sample (not centrifuged) (pyr. I=0·7 % w/v):					Commercial sample is 0·89 times as toxic as standard
0·187	0·272	28·1	4·420	75	
0·339	0·530	45·1	4·877	70	
0·672	0·827	68·2	5·473	88	
1·271	1·041	86·2	6·089	60	

* B=badly affected; M=moribund; D=apparently dead; based on three replicates of approximately twenty-five insects each.
† Discarded as introducing heterogeneity.
χ^2 for colourless standard for $n'-2=1$ is 0·304.
χ^2 for commercial sample $n'-2=2$ is 0·607.
χ_b^2 for parallelism=0·906. Departure from parallelism not significant.

material gave highly heterogeneous data (see Table 11 and Fig. 9).

It is difficult to draw precise conclusions from these three experiments, but the results show that excess presence of sludge in pyrethrum-in-oil preparations may render the technique of application difficult, and introduce heterogeneity into the results. Further work is required to ascertain how preparations containing large amounts of sludge may be applied to give homogeneous data. Although the

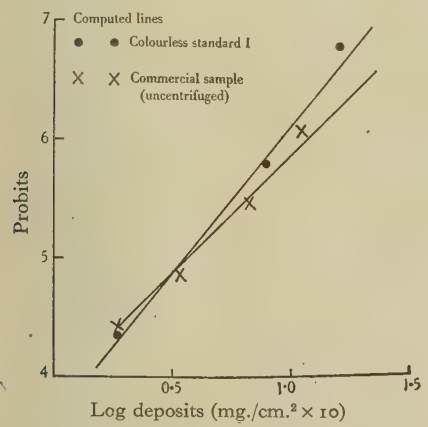

Fig. 8. Film technique to compare the toxicities of pyrethrum-in-oil preparations. Toxicities of colourless standard I and a commercial product (uncentrifuged) (11/12/41).

(*c*) *Effect on toxicity of variation of the constituents of pyrethrum-in-oil preparations*

No biological evaluation can be undertaken without a standard of reference, and the choice of such a standard is not easy. Experience has shown that the toxic and paralysing properties of pyrethrum are of an unique kind and that only a standard containing pyrethrum extract is of value The preparation of such a standard was undertaken (Martin, 1943). To ascertain whether the method of preparing solutions of pyrethrum extracts in oil, or the amount or nature of their non-pyrethrin constituents were a primary consideration in the insecticidal effect, the following standard solutions were prepared in different specifications and assayed biologically:

Coloured standard, prepared 10 Jan. 1941 from petroleum ether extract of pyrethrum flowers.
Oil—probably Wakefield half-white.
Colour—yellow. Sp. gr. 0·84 (approx.). Antioxidant—commercial preparation.
Pyrethrin content I=0·79 % w/v; II=0·96 % w/v; total 1·75 % w/v.
Total resin content (including pyrethrins)=7·6 % w/v.

Colourless standard no. I, prepared 8 May 1941 from petroleum ether extract of pyrethrum flowers mixed with decolorizing charcoal.
Oil—Wakefield half-white.
Colour—pale yellow. Sp. gr. 0·867. Antioxidant—pyrocatechol 0·25 % w/v in ethyl ether 2·5 % w/v.
Pyrethrin content I=0·80 % w/v; II=0·61 % w/v; total 1·41 % w/v.
Total resin content (including pyrethrins)=4·1 % w/v.

TABLE 11. *Film technique to compare the toxicities of pyrethrum-in-oil preparations*

Comparison between colourless standard I and a commercial sample.
Substratum: Whatman filter paper no. 544. Test subject: adult *Tribolium castaneum* Hbst. (25 Feb. 1942).

Deposit mg./cm.²	log (deposit × 10)	$B+M+D$ corrected for control %	Marks %	Experimental probits on marks	No. of insects used	Notes
Colourless standard I (1) (pyr. I = 0·8 % w/v):						
0·875	0·942	23·4	23·8	4·287	58	$\chi^2 = 6·80$ slight heterogeneity
1·22	1·086	38·0	32·2	4·538	50	
1·690	1·228	64·9	60·4	5·264	62	$\chi_b^2 = 1·532$
2·60	1·415	98·3	97·4	6·943	61	
Commercial sample (centrifuged) (2) (pyr. I = 0·8 % w/v):						$M = 0·04$; Antilog $M = 1·1$
0·749	0·874	26·2	23·7	4·284	63	
1·05	1·021	24·8	26·2	4·363	55	
1·381	1·140	64·3	62·0	5·306	55	
2·22	1·346	94·5	85·9	6·076	57	$\chi^2 = 5·13$. Not heterogeneous
Commercial sample (not centrifuged) (3) (pyr. I = 0·8 % w/v):						
0·781	0·893	49·1	42·0	4·798	61	
1·10	1·041	52·7	59·6	5·243	46	Not analysed
1·535	1·186	77·2	70·6	5·542	59	
2·35	1·371	100	96·0	6·751	57	

Marks: not affected = 0; slightly affected = 2·5; badly affected = 5; moribund = 7·5; dead = 10. Three replicates of approximately twenty insects each.

Colourless standard no. II, prepared 30 Apr. 1942. Extract as no. I.
Oil—Shell 24210.
Colour—pale yellow. No antioxidant.
Pyrethrin content I = 0·80 % w/v; II = 0·84 to 1·0 % w/v.
Total resin content (including pyrethrins) = 4·6 % w/v.
The word 'standard' is not used in the sense of being a final comparative test solution, but one prepared in a specified way and containing known amounts of constituents.

Comparisons were first made between a laboratory production (colourless standard solution I), and commercial samples, the pyrethrin content of which had been estimated to determine whether there was any gross difference in the toxicities when these were based on the pyrethrin content.

Experiment 11 *Dec.* 1941. Comparison between colourless standard solution I, and a commercial sample containing much sludge. Substratum: Tricoline.

This experiment was mentioned in connexion with the effect of sludge on toxicity (p. 272), where it was shown that the relative toxicity of the laboratory preparation and the commercial sample were in close agreement with the ratio of the pyrethrin content.

To determine whether these results are affected by the substratum an experiment was done (see below) using thin hardened filter paper Whatman no. 544.

Experiment 25 *Feb.* 1942. Comparison between colourless standard solution I, a commercial sample containing sludge and a similar sample with the sludge centrifuged out. Substratum: Whatman no. 544 filter paper. Each solution was tested at 0·8 % w/v of pyrethrin I (see Table 11 and Fig. 9).

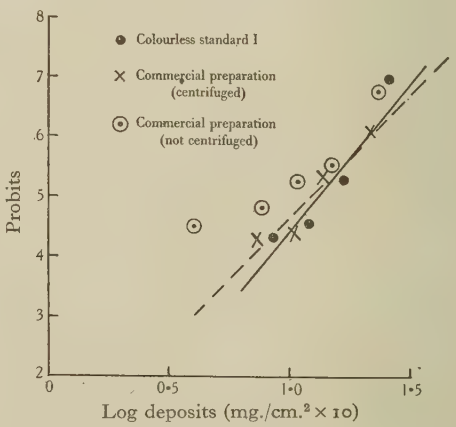

Fig. 9. Film technique to compare the toxicities of pyrethrum-in-oil preparations colourless standard I and a commercial preparation. (Based on marks $N = 0$, $S = 2·5$, $B = 5$, $M = 7·5$, $D = 10$) (25/2/42).

The sample containing sludge gave very heterogeneous results and could not be compared with the other two. The data for the colourless solution I and the centrifuged commercial sample were on the margin of heterogeneity, due almost certainly to the effect of the highest point in the case of the standard

and the lowest in the case of the commercial sample, but the slope of the regression lines is not significantly different although the lines cross:

$$M = \log \frac{\text{potency of centrifuged commercial sample}}{\text{potency of colourless laboratory solution I}}$$

$$= 0{\cdot}0404 \pm 0{\cdot}0019,$$

thus the centrifuged commercial sample is $\times 1{\cdot}097$ as potent as the standard. Based on their pyrethrin I content the samples should have the same toxicity and it is doubtful whether the observed difference is significant.

In this experiment an attempt was made to render the data more homogeneous by awarding marks to the various categories. The unaffected class was given no marks, each of the slightly affected insects 2·5, of the badly affected 5·0, of the moribund 7·5, and of the apparently dead 10. The marks were summed and the percentage of the total possible determined and converted to probits. In general this procedure was not very useful; in this case there was a slight improvement, but the figures for the commercial sample containing the sludge were still so heterogeneous as to be useless, though this may be due to a pronounced break at the lower end of the line. The slope of the regression line of the centrifuged commercial sample was again less steep, but not significantly so, than the standard and there was a marked tendency towards a break in the line at 4 probits.

Although no satisfactory figures were obtained for the toxicity of the material containing much sludge when filter paper was used as a substratum, it appears from the above two experiments that the relative potency of the laboratory preparation and the centrifuged commercial preparation is closely correlated with their pyrethrin content, and that other differences in constitution and physical properties that may exist have no significant effect on toxicity under the conditions of the experiment.

Comparison between coloured standard solution and colourless standard solution I

Experiment 3 *Feb.* 1942. Substratum: Whatman filter paper no. 544. The specifications are set out on p. 273. A concentration of 0·8% pyrethrin I was taken for each and the deposits (mg./cm.^{2}) were varied between 0·168 and 2·486. Log deposits were plotted against probits. There was a very slight heterogeneity in the data for the colourless standard, but it was not enough to invalidate the calculation of the relative potency. It was largely due to the lowest point and was almost entirely eliminated when this value was discarded. Data where the probits are below, e.g. 4 or above 6·3, might with advantage be discarded, as the numbers of insects used are generally insufficient for their accurate determination (see Table 12 and Fig. 10). Statistical analysis gave the figures inserted in the last column and at the foot of Table 12. A comparison of these results with those given by the chemical analysis is shown in Table 14 (p. 277).

These results indicate that the differences between the two solutions in method of preparation, colour, antioxidant and soluble resin content had no significant effect on toxicity; the relative potencies were more closely related to the contents of the total pyrethrins rather than to the contents of pyrethrin I.

Comparative toxicities of colourless standard solutions I and II and a commercial product

Experiment 5 *May* 1942. Substratum: Whatman filter paper no. 544. In view of the above results for the coloured and colourless standards and the possibility that the difference in their toxicities might be due to the content of pyrethrin II being higher in the coloured preparation, a series of tests was made, using a newly prepared colourless standard containing no antioxidant and a fresh sample of the commercial product. They were also compared with the colourless standard I used in the above trials. After chemical analysis both the colourless standards were made to contain 0·8% w/v of pyrethrin I, but, inadvertently, the commercial product was diluted to and tested at 0·92% w/v of pyrethrin I (Wakefield half-white oil being used as diluent in all cases). The data (probits *v.* log deposit) were homogeneous, but no results below a 50% value, in badly affected, moribund and dead were obtained, owing to the insects being of a lower resistance than those from previous cultures. As in practically all previous tests the regression line for the commercial product had a less steep slope than those given by the standard preparation. The departure from parallelism was not statistically significant, but the frequency with which in our tests the slope for the regression line of the commercial samples is less steep than that of the standard preparations, leads to the deduction that some factor in the former accentuates toxicity at lower deposits and that the effect becomes proportionally less as the weight of the deposit is increased. Again, there may be a break at the bottom part of the regression line for the commercial sample (see Table 13 and Fig. 11).

The results were as follows:

(*a*) The colourless standard II (prepared 30 Apr. 1942) is $\times 1{\cdot}275 \pm 0{\cdot}079$ as toxic as the colourless standard I (prepared 28 May 1941). Both solutions tested at a content of pyrethrin I = 0·8% w/v.

(*b*) The commercial product at 0·92% w/v pyrethrin I is $\times 1{\cdot}057 \pm 0{\cdot}0022$ as toxic as the colourless standard II at pyrethrin I = 0·8% w/v.

Experiment (*a*) emphasizes the previous finding that the determination of the pyrethrin I content alone is not a satisfactory index of the toxicity of pyrethrum-in-oil preparations.

TABLE 12. *Film technique*

Comparison between the toxicity of colourless standard I and a coloured standard. The effect on toxicity of constituents other than the pyrethrins.

Substratum: Whatman filter paper no. 544. Test subject: adult *Tribolium castaneum* Hbst. (3 Feb. 1942).

ml. in reservoir	log reservoir content	Deposit mg./cm.^{2}	log (deposit × 10)	$B+M+D$* %	Experimental probit	No. of insects	Value of $M = \log \frac{\text{potency of B}}{\text{potency of A}}$
A. Colourless standard I (pyr. I = 0·8 % w/v):							
(1) 1	0·000	0·469	0·671	6·8	3·5091	60	
(2) 2	0·301	0·922	0·964	25·3	4·3349	64	
(3) 4	0·602	1·802	1·256	67·2	5·4454	62	$M = 0·09$
(4) 6	0·778	2·633	1·421	97·0	6·8808	67	Antilog $M = 1·23$
B. Coloured standard (pyr. I = 0·79 % w/v):							
(5) 1	0·000	0·600	0·778	15	3·9636	61	Omitting (1)
(6) 2	0·301	1·148	1·060	62·0	5·3055	67	$M = 0·106$
(7) 2	0·301	1·242	1·094	57·6	5·1917	60	Antilog $M = 1·276$
(8) 4	0·602	2·486	1·396	100	—	60	

* B = badly affected; M = moribund; D = apparently dead. % corrected for control. Based on three replicates of twenty insects each.

χ^2 for colourless standard I for $n' - 2 = 2 = 7·054$. (Slight heterogeneity.)

χ^2 for colourless standard I for $n' - 2 = 1 = 4·041$ (omitting value (1)). (On margin of heterogeneity.)

χ^2 for coloured standard for $n' - 2 = 2 = 2·857$. (Not heterogeneous.)

$\chi_b^2 = 1·091$, χ_b^2 omitting (1) = 0·00034 (departure from parallelism not significant).

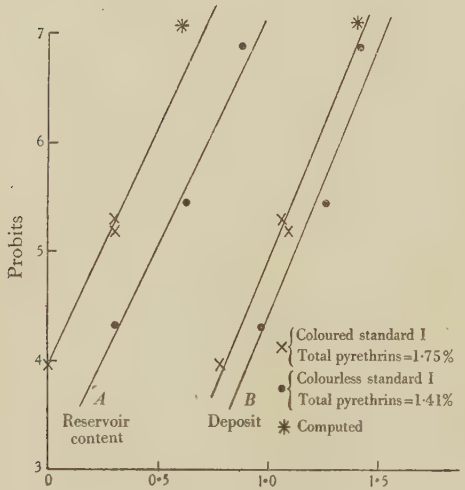

Fig. 10. Film technique to compare the toxicities of pyrethrum-in-oil preparations. Comparison of toxicities of two pyrethrum-in-oil standards (3/2/42). The figure also demonstrates that the reservoir contents of two pyrethrum-in-oil products of different composition cannot be taken to be strictly correlated with the deposits per unit area, and that the differences can be sufficiently large as to affect the determination of their relative potencies. Curves *A* show probits plotted against log reservoir contents and *B* plotted against true log deposits. The latter express the relative potency much nearer the values given by the chemical determination of total pyrethrin content.

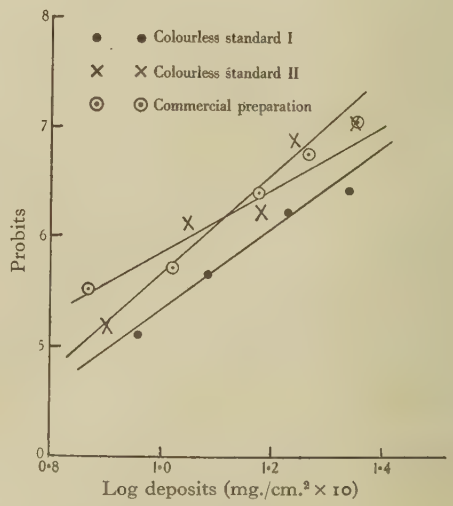

Fig. 11. Film technique to compare the toxicities of pyrethrum-in-oil preparations. Toxicities of two standards and a commercial preparation (5/5/42).

TABLE 13. *Film technique*

Comparison between colourless standards I and II and a commercial preparation. The effect on toxicity of constituents other than the pyrethrins.

Substratum: Whatman filter paper no. 544. Test subject: adult *Tribolium castaneum* Hbst. (5 May 1942). Based on three replicates of approximately twenty insects each.

Mean deposit mg./cm.²	log (mean deposit × 10)	*B*+*M*+*D* %	Experimental probit	No. of insects	Notes
(1) Colourless standard no. II (pyr. I=0·8 % w/v):					
0·796	0·901	56·7	5·169	52	
1·109	1·046	86·4	6·099	58	
1·513	1·180	88·8	6·216	60	χ^2 (for $n'-2=4$)=3·913.
1·757	1·240	97·0	6·881	39	Data not heterogeneous.
1·933	1·286	100	—	39	
2·246	1·351	98·0	7·054	60	
(2) Colourless standard no. I (pyr. I=0·8 % w/v):					
0·915	0·961	53·8	5·095	56	
1·215	1·085	73·9	5·640	56	χ^2 (for $n'-2=3$)=1·994.
1·713	1·234	89·0	6·227	61	Data not heterogeneous.
2·204	1·343	92·3	6·426	59	
2·670	1·427	100	—	59	
(3) Commercial preparation (pyr. I=0·92 % w/v):					
0·739	0·869	72·4	5·496	58	
1·051	1·022	76·3	5·716	57	χ^2 (for $n'-2=3$)=2·103.
1·498	1·176	91·8	6·392	55	Data not heterogeneous.
1·843	1·266	96·0	6·751	56	
2·257	1·354	98·0	7·054	60	

χ_b^2 for (1) and (2)=0·1256. Lines do not depart significantly from parallelism.

$$M=\log\frac{\text{potency of (2)}}{\text{potency of (1)}}=\bar{1}\cdot8942\pm0\cdot0325=\text{antilog }0\cdot784.$$

I.e. Colourless standard II is 1·275 times as potent as colourless standard I.

χ_b^2 for (1) and (3)=2·6467. Departure from parallelism is not significant.

$$M=\log\frac{\text{potency of (3)}}{\text{potency of (1)}}=0\cdot0242\pm0\cdot039=\text{antilog }1\cdot057.$$

Commercial preparation is × 1·057 as toxic as standard II.

Comparison of insecticidal results and with those of chemical analysis

A primary purpose in all these tests was the comparison of the biological and chemical techniques as means of evaluation, but it was only possible to accumulate preliminary data. The chemical analytical data for the standard preparations were compared with those given by the biological tests. Table 14 expresses them in concise form.

With the samples used in the direct spraying technique (p. 269) it made no significant difference whether the loss of activity was judged by the contents of pyrethrin I or by the total pyrethrins. This indicates either that under the conditions of the experiment the two pyrethrins deteriorate at the same rate or that pyrethrin II is not an important factor in their toxicity. Table 14 (*a*, *b*, *c*) shows that the total pyrethrin content of pyrethrum-in-oil preparations gives a slightly closer approximation to

TABLE 14. *Comparison of insecticidal results with chemical analysis*

	Pyr. I %	Pyr. II %	Total %	Ratio Pyr. I values	Ratio total pyrethrins	Relative toxicities
(*a*) Coloured standard	0·79	0·96	1·75	*a*/*b*=1·0	*a*/*b*=1·24	*a*/*b* 1·23 −1·276
(*b*) Colourless standard I	0·80	0·61	1·41			
(*c*) Colourless standard II	0·80	0·84–1·0*	1·64–1·80	*c*/*b*=1·0	*c*/*b*=1·16–1·28	*c*/*b* 1·275±0·079
(*d*) Commercial prep. I (centrifuged)	0·80	—	†	*d*/*b*=1·0	—	*d*/*b* 1·097±0·0048
(*e*) Commercial prep. II	0·92	—	†	*e*/*c*=1·15	—	*e*/*c* 1·057±0·0022

Note. Pyrethrin I determined by Wilcoxon method, pyrethrin II by Seil method.

* Difficulty in giving true pyrethrin II value because of fatty material present.

† Pyrethrin II values not determined.

(*a*) and (*b*) contain antioxidant, (*c*) no antioxidant.

the true relative toxic value than the pyrethrin I content taken by itself. In the case of the commercial preparations the pyrethrin II values were not determined, nor was it known whether activators were present. An evaluation based upon pyrethrin I alone would not lead with these particular solutions to any grave error, nevertheless it is clear that a slight discrepancy exists between the biological and the chemical results, which emphasizes the need for a chemical method for accurately determining pyrethrin II, and for the final test preparation to be standardized if possible, with respect to both pyrethrins.

The experiments also indicate that the effect of different media upon the relative toxicities of the two pyrethrins needs determination, and Martin (1943) undertook the preparation of two concentrates, one rich in pyrethrin I and the other in pyrethrin II.

Results and conclusions

Techniques

The characteristics of a suitable test insect and the rearing and standardization of *Tribolium castaneum* Hbst., the species chosen, are discussed (p. 262).

The spraying apparatus was that described by Potter (1941). For direct spraying, minor modifications of the technique previously adopted for aqueous sprays were made, and the range of concentrations and deposits suitable for use with pyrethrins in heavy oil media determined. The film technique for testing insecticides and the properties of a satisfactory substratum for use with it are discussed. Tricoline and thin hardened filter paper (Whatman no. 544) were finally adopted. Time of exposure of the insects on the films was fixed at 5 days and a suitable range of deposits and concentrations determined.

The method of examination and classification of the insects after treatment is described. Earlier examinations were made at laboratory temperatures, later ones on a specially constructed warm plate which valuably modified the method (p. 263).

Usual statistical technique was employed for the figures of experiments involving the relationship of probits to log concentration of poison *or* log weight of deposit. For experiments involving the relationship of probits to *both* log concentration and log deposit, a special technique was devised by Mr D. J. Finney by means of which probit planes could be constructed.

The relationship between the amount of fluid in the reservoir of the apparatus and the amount deposited was nearly but not quite linear, whether the spraying was on to a flat disk or on to a Petri dish. The deposit per unit area for the same volume in the reservoir was greater on the disk.

Very slight changes in the physical properties of the spray fluid may have a marked effect on the weight of deposits. Viscosity relationships are important in this connexion, but an attempt to correlate weight of deposit with physical properties was inconclusive. It is necessary to check deposits by weighing whenever possible.

To compare direct spraying with the film technique, experiments were made varying the weight of deposit while keeping the concentration constant, and vice versa. The figures were analysed by the method of probit planes; these were parallel for the two methods. Using a textile fabric as substratum, direct spraying gave somewhat higher kills than the film technique for a given concentration or deposit. Increasing the concentration was more effective than increasing the deposit. There was no evidence that one technique was significantly better than the other in the degree of homogeneity of the results.

Application of techniques

The loss of activity of the pyrethrins in two different media under a given set of conditions was determined by the direct spray technique. It was possible to distinguish with certainty a loss of activity of 10% over the range of concentrations tested: smaller differences were not investigated. The figures obtained agreed well with the results of chemical analysis.

Using the film technique, the influence of the substratum on the relative effectiveness of increases in concentration and deposit was studied. With filter paper (Whatman no. 544) the data were satisfactorily fitted by a probit plane, but, contrary to earlier results with a textile fabric, increases in weight of deposit had a slightly greater effect on toxicity than increases in concentration. This result needs confirmation. With a Tricoline substratum, the data were closely fitted by a probit plane and the effect of increases in concentration was greater than that of increases in deposit, in agreement with results obtained with another textile fabric.

A commercial preparation of pyrethrins which contained much sludge was compared by the film technique with the same preparation from which the sludge had been removed by centrifuging and with a colourless laboratory preparation free from sludge. The sludge tended to interfere with the working of the technique by clogging the atomizing apparatus but, under the conditions of experiment, it had no great effect on toxicity. Further work on this point is desirable.

Three standard pyrethrum-in-oil preparations, brief specifications for which are given, were compared by the film technique with one another and with commercial pyrethrum insecticides to determine the effect on toxicity of the method of preparation, the percentage of soluble resin, and the presence or absence of an antioxidant. Of the various constituents examined, apart from the oil, only the pyrethrins had a measurable effect on toxicity. The biological assessment agreed well with the results of

chemical analysis but correlation was closer with the percentage of total pyrethrins than of pyrethrin I, a result which stresses the importance of finding a satisfactory method of determining pyrethrin II.

We are indebted to Drs J. T. Martin and S. H. Harper for help during the course of this work and for determinations of the pyrethrins, and to Mrs Marjorie Piggott for her assistance in carrying out the spraying trials. We have also had much help from Mr D. J. Finney on the statistical side of our work, and our indebtedness to him is gratefully acknowledged.

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A film technique for the biological evaluation of pyrethrum-in-oil insecticides for use against stored product insects in warehouses

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(With 5 Text-figures)

There is need for a technique whereby a given insecticidal preparation can be compared with a standard containing 0·8 % (w/v) pyrethrin I in a heavy white oil and pronounced biologically equal or inferior to the standard within known and reasonable limits. A film method was investigated, *Tribolium castaneum* beetles being used as the test insects.

Numerous factors which may influence the kill achieved are discussed. The results of preliminary work, planned to help in the standardization of some of these factors, are summarized.

Several materials were tested to ascertain their suitability for reception of the insecticidal film and, as thin greaseproof paper showed promise, its possibilities were explored. Although 0·3 % pyrethrin I could be distinguished from 0·4 %, 0·5 % could not be distinguished from 0·8 %, and the results throughout were very heterogeneous.

Woven Nylon proved more satisfactory, since the test just failed to establish a significant difference between 0·6 and 0·8 % pyrethrin I. The origin of a pink coloration of the substrate, when *T. castaneum* beetles were confined on Nylon sprayed with pyrethrum in oil, was investigated.

Whatman filter paper no. 544 proved the best substrate, and a comparative test on it will distinguish 0·65 % pyrethrin I from the standard 0·8 %; the limit could probably be raised to 0·7 %.

The influence of several factors on the homogeneity of the results was investigated, and the importance was shown of allowing beetles time to recover from the mechanical shock inherent in the method of counting into batches.

Directions are given for carrying out the film technique for comparing preparations of relatively high pyrethrin content. The method does not measure the direct spray or fumigant action of the insecticide.

The technique described is compared with the Peet-Grady method for evaluating liquid household insecticides of low pyrethrin content. A simplified, but less accurate, design for the test is indicated.

Introduction

Much information upon the chemistry, evaluation and biological effects of pyrethrum exists in the literature. A new departure was the introduction by Potter (1935) of the use of mists and films of pyrethrum extract in heavy oil in warehouses containing dried fruits infested by the Phycitid moths, *Plodia interpunctella* Hb. and *Ephestia elutella* Hb. A similar method was recently tried with encouraging results for the reduction or elimination of the resident population of insects in buildings housing other stored produce, particularly grain and grain products. Pyrethrum is expensive, and must be imported into this country. The Pest Infestation Laboratory of the Department of Scientific and Industrial Research is therefore investigating the economic use of this and similar materials. One way in which the consumption of pyrethrum may be reduced is the inclusion in insecticides of substances which may act by aiding penetration of the pyrethrum through the insect cuticle. The principles underlying the mode of action of such activators are being elucidated by Hurst (1940, 1941). If effective activators are present, chemical estimation of the quantity of basic principle, e.g. pyrethrins, in the mixture is not a direct indication of the toxicity of the mixture and a biological test is necessary.

There was need to devise a method whereby (i) the influence of different factors upon the efficiency of an insecticide could be assessed, and (ii) the relative toxicities of insecticides could be compared under controlled conditions. Such a test would be a starting-point for the general experimental work and, as an extension of the second item, it was very desirable to have a test whereby a given insecticidal preparation could be pronounced biologically equal or inferior to a standard within known and reasonable limits.

So that the laboratory test should resemble practice, a method of application of the insecticide was used which involved atomization. Exposure to the insecticide could then be direct by spraying the insects, or indirect by spraying a suitable surface and placing the insects upon the resultant toxic film. The film method was selected for immediate investigation, since Potter (1938) has shown that films of pyrethrum in a heavy oil are toxic over a limited period to certain species of insects common in warehouses and because, in practice, it is often impossible to reach with a direct spray many insects concealed in stored goods, between boxes, etc., or in crevices in the fabric of a building.

A solution of 0·4% (w/v) pyrethrin I in Shell Oil P. 31, a heavy white oil of the same specification as Shell Oil 24210 (Potter, 1935), was used as the standard insecticide, and a test to distinguish 0·3 from 0·4% pyrethrin I seemed a good objective in the first instance. Some time after the start of the work, the Department of Scientific and Industrial Research, on the advice of Prof. J. W. Munro and Mr J. C. F. Fryer, adopted as the specification of a standard spray for use against coleopterous pests in warehouses a solution of 0·8% pyrethrin I in a heavy oil. Clearly this concentration ought to be employed in the laboratory tests rather than 0·4%; the early experiments were therefore undertaken with a standard different from that of later tests. The source of pyrethrum was a commercial concentrate in heavy oil, diluted as required. The concentration of pyrethrin I was determined by Dr J. T. Martin using a modified Seil method and was later redetermined by him by a modified Wilcoxon-Holaday method. The second method was the more accurate, and as soon as Dr Martin's figures were available the dilution was changed. More recent samples of the extract were similarly assayed chemically for pyrethrin I content at the Imperial Institute, South Kensington, S.W. 7.

The chief difficulty in the investigation was the discovery of a surface upon which the insecticide could be sprayed in a series of known deposits so that the corresponding mortalities of beetles exposed to them would cover the range from 0 to 100%. As soon as a promising material, a thin grade of greaseproof paper, was discovered, the possibility of using it in a film test was explored and the trial of other surfaces postponed. The knowledge thus gained was valuable and, when the limitations of the material were realized, was used to develop a technique with woven Nylon and, finally, a hardened filter paper as the standard surface. This report presents a summary of the data obtained during the testing of various materials as substrates for toxic films, and traces the development of a method of biological evaluation of insecticides of pyrethrum in heavy oil to a stage where a satisfactory technique could be described. By this technique pyrethrum preparations may be compared with a standard containing 0·8% pyrethrin I in Shell Oil P. 31 and the limits of the test fairly closely defined. A brief general account of the work was published by Parkin (1942). A few inconsistent results were obtained which cannot yet be explained. With more knowledge and experience the procedure of the test may be modified and reasons found for occasional, aberrant results. The test measures the film effect only of an insecticide and accounts for neither spray nor possible fumigant action, both of which require the development of independent methods of evaluation. With the information gained in devising the present film test the establishment of suitable techniques for evaluating the spray and fumigant effects should not be difficult.

Factors influencing mortality

Before starting experimental work, a list was made of the factors which might influence the mortality of beetles exposed to a toxic film. Some of these

were arbitrarily standardized; others could not be fixed until after preliminary investigation.

A. *Breeding the test insects*

(i) *Species. Tribolium castaneum* Herbst. beetles were chosen as the test insects for their relatively high resistance to insecticides generally, for the ease with which they can be bred and handled in large numbers, and for their inability to climb glass. It might be advisable to consider the use of several species of insects in this type of test.

(ii) *Stock.* The Laboratory's general stock was accepted as the parent stock.

(iii) *Food.* All cultures were reared on wholemeal flour made from North Manitoba no. 1 wheat.

(iv) *Temperature and humidity.* Insect stocks were bred in a constant temperature and humidity room maintained at 24° C. and 70% R.H.

(v) *Density of population.* This was kept as low as possible in relation to the numbers of beetles required and space available for breeding.

(vi) *Sex.* Nothing is known of the relative resistance of the sexes of *Tribolium* to contact insecticides.

(vii) *Age.* The best age of beetles for experimental use should be determined, but insects 3–5 weeks old proved satisfactory. For the method in use at this Laboratory to produce a steady supply of beetles of known age, see p. 290.

B. *Treatment of insects before exposure to film**

(viii) *Temperature and humidity.* By testing at 24° C. and 70% R.H. the need for conditioning the beetles was avoided.

(ix) *Removal from food.* In view of reports by workers on other insects, e.g. Craufurd-Benson (1938), that a starvation period up to 24 hr. tended to make for greater uniformity in resistance, the beetles were starved overnight before use.

C. *Conditions during exposure to film*

(x) *Substrate to receive film.* Much experimental work was directed to the discovery of a suitable surface upon which to deposit the film of insecticide.

(xi) *Concentration of insecticide.* Two standards were used; in the earlier work 0·4%, and in the later 0·8% pyrethrin I in Shell Oil P. 31. Different resin contents may affect the toxicity of the insecticide, but nothing was known of the importance of this point. The preparation of a chemically standardized insecticide was not completed by Dr Martin in time for use to be made of it in this work.

(xii) *Carrier.* The toxicity of pyrethrum may vary according to the physical characteristics, e.g. viscosity, of the oil in which it is dissolved (Tattersfield & Potter, 1943) and different oils may themselves have different insecticidal properties. A heavy white oil, Shell Oil P. 31, was used throughout as diluent for the concentrate.

(xiii) *Deposit of insecticide.* A suitable range of deposits was determined for each type of surface. Each deposit should be weighed.

(xiv) *Temperature and humidity.* The temperature at the time of exposure may seriously affect the final kill, whereas the influence of humidity should be less noticeable except at extremes: 24° C. and 70% R.H. were used throughout the investigation.

(xv) *Age of film.* The interval between deposition of the film and exposure of the beetles must be determined experimentally with reference to nos. (x), (xi), (xiii) and (xvi) for each change in the method of the test.

(xvi) *Period of exposure.* The period of exposure of the insects to the film must be determined experimentally with reference to nos. (x), (xi), (xiii) and (xv).

(xvii) *Method of exposure.* The insects were tipped on to the centre of the treated surface and covered immediately with an inverted glass funnel, 6 cm. diam., with the edge ground flat to prevent their escape. Thin materials like paper were laid on a sheet of glass so that the funnels might bed down evenly. Glass rings were used later as an alternative to funnels and would clearly be advantageous, when insecticides contain respiratory poisons or other volatile compounds.

(xviii) *Crowding.* A 6 cm. funnel or ring was used to confine fifty beetles without apparent overcrowding.

(xix) *Light intensity.* Darkness or lighting of low and even intensity is advisable during the exposure period. The intensity of the lighting, through its effect on the general activity of the beetles, may influence the mortality.

D. *Conditions after exposure to film*

(xx) *Container.* In the first half of the work the periods of exposure of beetles to the films did not exceed 24 hr. and the beetles were then removed for observation to 4 oz. glass jars of approximately 2 in. internal diameter and 2¾ in. deep. A 1 in. hole was punched through each screw cap and underlying waxed cardboard disk and a small circle of muslin inserted between.

(xxi) *Foothold in containers.* A disk of Whatman no. 1 filter paper covering the bottom of the jar markedly reduced the number of beetles classified as affected, without causing significant change in mortality. The paper did not affect the death-rate of control insects, and a disk was put in all containers to which beetles were removed from the toxic films for observation.

(xxii) *Temperature and humidity.* A preliminary experiment showed that mortality tended to rise as the temperature of storage fell. In all comparative work the insects were maintained at 24° C. and 70% R.H.

* See also the effect of disturbance during counting (p. 286).

(xxiii) *Observation period.* Combined observations on some 6000 untreated beetles, 3–5 weeks old at the start of the experiments, are shown in Fig. 1. The observation period must not exceed 12 days and should preferably be less. Counts of dead and affected beetles were made at various intervals up to 10 days, when the expected control mortality was about 5%.

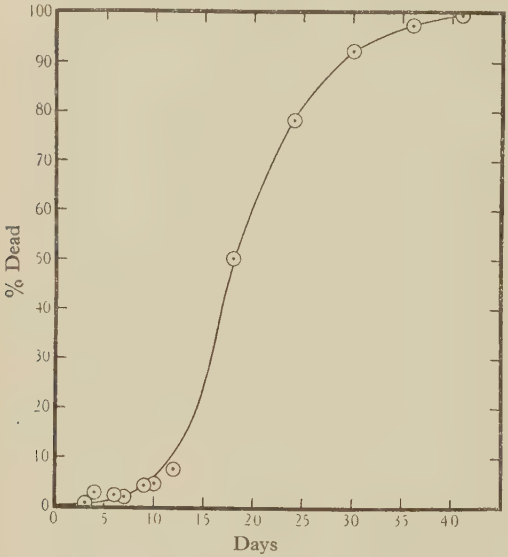

Fig. 1. Relation between mortality and period of starvation of *T. castaneum* beetles, 3–5 weeks old at the start, and kept at 24° C. and 70% R.H.

Preliminary tests with various substrates

Numerous preliminary experiments were directed to the discovery of a material upon the surface of which the insecticide could be sprayed, so that a suitable range of mortalities could be covered by a convenient series of deposits.

The square tower constructed by Potter (1941) was used for deposition of the films, but modified from time to time to gain greater control of the deposits. Inflexible materials, e.g. 3 in. squares of glass, plywood or cardboard, were supported directly under the tower. Flexible materials like paper and cellophane were cut into 4 in. squares, in one corner of which a small hole was punched, so that they could quickly be hung for weighing on a wire hook on a balance arm. The prepared squares were handled with forceps and kept in a dust-proof cover in the laboratory to equilibrate with local conditions of relative humidity. A square was weighed and laid quickly between two cardboard masks, each $4\frac{1}{2}$ in. sq. with a central aperture 3 in. sq. The paper, thus supported, was placed under the tower, sprayed, removed from the masks and immediately reweighed to enable the deposit per unit area to be computed. To prevent seepage from the sprayed to the unsprayed area, the edges were trimmed with scissors so that only the 3 in. sq. sprayed area remained. Several of these, each covered centrally by a funnel, were laid on a sheet of glass and transferred to the constant-temperature room.

At selected periods up to 24 hr. after spraying, batches of fifty *T. castaneum* beetles were tipped on to the squares and the funnels quickly replaced. After exposure up to 24 hr. the beetles were removed to jars for periodic observation of mortality. Batches of control insects were treated similarly, except that they were exposed upon unsprayed squares.

Finely ground glass plates and cellophane proved unsuitable as substrates, because the very small deposits required to avoid a 100% kill could not be applied with sufficient accuracy. Potter (1938) indicated that *T. castaneum* beetles are killed by short exposure to a light deposit of pyrethrum spray on Canadian hemlock. Our experiments with birch plywood and Scots pine showed that the deposits needed to obtain kills high enough for comparative work were too great to be practicable. Different grades of filter paper, Bristol board and glazed cardboard were too absorbent and high deposits resulted in low kills.

A fruit wrapping paper, a vegetable parchment, and thick and thin greaseproof wrapping papers were tried, the last three being obtained through the help of Mr Wm. Whyte, Greaseproof Paper Mills, Ltd., 46 Mansell Street, London, E. 1. Thin greaseproof paper showed promise, and more detailed work was carried out to develop a film method for the biological evaluation of pyrethrins, using this paper as the standard substrate.

Experiments with thin greaseproof paper

Using the square spraying tower fitted with a special nozzle (Potter, 1941), 12–15 deposits were applied by the method described. Preliminary experiment showed the useful range of deposits to be 2–4 mg./sq. in. for 0·4% or 1·8–2·4 mg./sq. in. for 0·8% pyrethrin I in Shell Oil P. 31. Twenty-four hours after deposition of the films, batches of fifty beetles were put under funnels on the papers and exposed for 24 hr. The numbers of dead beetles were recorded 7 and 10 days after removal to the 4 oz. jars.

The percentage kills after 10 days were corrected, if necessary, for control mortality and, with the corresponding deposits, were analysed by Bliss's method (1935 *a*, *b*), whose notation is adopted here. In all experiments, the χ^2 test for goodness of fit of the experimentally obtained log dose-probit points to the regression lines showed the results to be very heterogeneous. Although the analysis of such heterogeneous data could be continued, to discover whether the regression lines differed significantly in position and slope, the heterogeneity was an evident disad-

vantage of the film test as described, especially as the values of χ^2 themselves varied greatly from experiment to experiment.

A significant difference ($P = <0{\cdot}001$) in the positions of the regression lines was determined in two tests comparing 0·4 with 0·3 % pyrethrin I. Another test just failed ($P = 0{\cdot}07$) to distinguish between 0·4 and 0·325 %. In view of the recommendation by the Department of Scientific and Industrial Research that 0·8 % pyrethrin I be used for the control of coleopterous pests of stored products, it was important to find the limit of the test using this higher concentration as the standard of comparison. Experiments showed that 0·6 and 0·5 % pyrethrin I could not be distinguished from 0·8 %. The probable explanation of the wider limits at the higher concentration is that the relation between concentration and toxicity, as measured by the deposit required for a 50 % kill, is not a straight line for this particular surface.

Although the limit of the assay using 0·4 % pyrethrin I on thin greaseproof paper could be regarded as satisfactory, the test was disappointing in relation to the use of 0·8 % pyrethrin I as standard. The failure of the test to reach expectation was due mainly to two factors: (i) the non-linear relationship between concentration and insecticidal power, and (ii) the variation in mortality of beetles between and within individual experiments.

The variation encountered seemed, from general experience with insects bred and tested under standard conditions, to be much greater than would be expected between batches of fifty. A part of the variation was probably due to the beetles receiving different doses of insecticide, which, in turn, was most likely to be caused by a lack of uniformity in the density of the film resulting from (*a*) unequal deposition of the film, because of poor distribution of the spray in the tower, and (*b*) unequal absorption of the liquid by different areas of the paper. It was hoped that a more even deposition of films might be achieved with the aid of a more efficient spraying apparatus (Potter, 1941). Unequal absorption of the liquid could be overcome only by the use of a more uniform material than thin greaseproof paper. Two substances which in large measure satisfied this criterion of uniformity were a woven Nylon fabric and filter paper, although the latter had previously been judged unsuitable for use with the short-exposure technique then employed.

Experiments with Nylon

Two experiments were made with woven Nylon fabric, obtained through the co-operation of Mr O. B. Lean, Messrs Imperial Chemical Industries, Ltd., Hawthorndale Laboratory, Jealott's Hill, Bracknell, Berks. The first was to discover a working range of deposits (1·5–3·5 mg./sq. in.) using 0·8 % pyrethrin I in Shell Oil P. 31. The results were employed in the planning of a second experiment to ascertain whether 0·6 % pyrethrin I in P. 31 could be distinguished from 0·8 % when tested as insecticidal films on this material.

The method was, in general, like that described earlier, but a major change was introduced, in view of results from contemporary experiments with filter paper, by placing the beetles on the newly sprayed surface and leaving them continuously in contact with the insecticidal film during the 9 days' period of observation. The spraying was done at laboratory temperature, but during exposure to the films the insects were kept at 24° C. and 70 % R.H.

The beetles were examined under a low-powered binocular microscope on the 3rd, 6th and 9th days, and counted into the four classes: normal, slightly

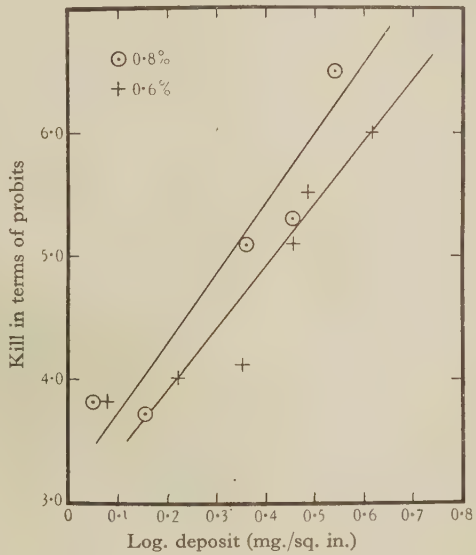

Fig. 2. Provisional regression lines showing the relation between mortality of *T. castaneum* beetles and deposit of 0·8 and 0·6 % pyrethrin I in Shell Oil P. 31 on Nylon. Exposure period 6 days.

affected, badly affected, and dead. Slightly affected beetles were those unable to walk normally but able to make co-ordinated, if somewhat hesitant, progressive movement; badly affected beetles were unable to make such progressive movement and often lay on their backs with legs and antennae twitching feebly.

Complete or partial recovery of some beetles was observed between the 6th and 9th days of exposure, which suggested that it would be preferable to rely on counts of dead insects only, until more is known of the reversible and irreversible effects of pyrethrum poisoning. The results of the mortality counts after 6 and 9 days were analysed by Bliss's method (1935 *a*, *b*), and the regression of mortality, expressed as probits, on log deposit is shown in Fig. 2 for both

concentrations. The slopes of the lines were much less steep with Nylon than with thin greaseproof paper. All sets of data exhibited heterogeneity, but the values of χ^2 showed a considerable improvement on those obtained with greaseproof paper.

The test just failed to differentiate between the two concentrations, when the positions of the lines were considered (see Table 1). Comparison of the slopes of the lines showed good agreement for parallelism.

TABLE 1. *Comparison of positions and slopes of regression lines for* 0·8 *and* 0·6% *pyrethrin I on Nylon*

	Exposure days	t	n	P (approx.)
Discrepancy in position	6	1·911	7	0·09
	9	1·849	6	0·12
Discrepancy in slope	6	0·643	7	0·53
	9	0·355	6	0·72

As more promising results were achieved with filter paper, the investigation of Nylon was not continued. Relatively heavy deposits of insecticide were required on filter paper, and it might be useful to have in reserve a material upon which considerably lighter deposits could be used, if occasion demanded.

When *T. castaneum* beetles were confined on the surface of sprayed Nylon, the fabric covered by the funnel became evenly and permanently pink within 24 hr. This colour did not develop beyond the perimeter of the funnel, or on unsprayed controls. Roth & Howland (1941) showed that *T. confusum* beetles secrete a volatile substance resembling *p*-benzoquinone in its general chemical and biological properties. A coloration like that caused by *T. castaneum* developed when a little quinone was suspended above Nylon under an inverted funnel, whether the material was untreated or sprayed with Shell Oil P. 31. Thus the beetles, when irritated by pyrethrum, secreted a volatile substance soluble in oil and similar to quinone in its colour reaction with Nylon. Under certain circumstances Nylon might prove a useful indicator of the presence of this secretion.

EXPERIMENTS WITH HARDENED FILTER PAPER

Early experiments with different grades of Whatman filter papers indicated that the soft types, such as no. 1, were too absorptive, so attention was confined to the thin, hard kind: Whatman no. 544 was chosen for test. Experiments showed that no. 544 was more absorbent than greaseproof paper, and that, even with relatively high deposits, a short-exposure period resulted in very low kills. A satisfactory range of mortality was obtained when beetles were placed on the newly sprayed filter papers and left in continuous contact with the film of insecticide during the period of observation. A preliminary experiment was undertaken to cover a wide field of investigation, so that an opinion could be formed of the merits of the technique employed, and sufficient experimental data could be acquired to indicate directions in which it might be improved. Subsequent experiments were directed towards achieving that improvement and determining the extent of variation still present in the technique finally adopted.

Insects, insecticides and deposits were prepared as described in the Nylon experiments: circles, 9 cm. diam., of no. 544 paper were used. The beetles, whilst still on the filter paper, were classified with the aid of a low-powered binocular microscope, the dead being removed at each examination.

A. *Preliminary experiment* (*no.* 49)

Twelve deposits, ranging from 1 to 19 mg./sq. in., were sprayed on to Whatman filter paper no. 544 for each of the concentrations, 0·8, 0·7, 0·6, 0·5, 0·4% pyrethrin I. Fifty beetles were tipped upon each paper, as soon as it had been sprayed, and were immediately confined under an inverted filter funnel. The beetles were counted into the four classes on the 3rd, 6th and 9th days of exposure. Four batches of beetles were kept under identical conditions on untreated filter paper for counts of control mortality. In all, 3200 beetles were used.

The regression lines representing the counts of dead beetles on the 9th day of exposure are shown in Fig. 3. Although the lines tend towards parallelism, there are considerable differences between the slopes and in the fit of the points to the respective lines. There is a group of points to the left of the figure which do not fit well on the lines drawn and indicate kills higher than those expected. Bliss (1935*a*) considers that such points may be treated separately from the remainder of the data. These 'breaks' in the lines were not apparent until the last count. The goodness of fit of the data to the regression lines is shown in Table 2.

TABLE 2. *Exp.* 49. *Fit of points to regression lines for exposure period of* 9 *days*

Conc. pyrethrin I %	χ^2	n	P (approx.)
0·8	2·72	1	0·10
0·7	2·07	2	0·40
0·6	13·37	3	0·005
0·5	5·21	3	0·14
0·4	23·40	3	<0·001

In spite of some low values of P, the data showed, on the whole, greater homogeneity than those for thin greaseproof paper. The sums of columns 2 and 3 of Table 2 gave $\chi^2 = 46{\cdot}77$ for 12 degrees of freedom, so that for the whole set of data $P = <0{\cdot}001$. This significantly low value of P, though due to only two of the sets of data, indicated that greater homogeneity in the results must be achieved before the test could be considered really acceptable.

The differences in slope of the regression lines are shown by comparison of the regression coefficients (Table 3).

TABLE 3. *Exp.* 49. *Slopes of the regression lines for exposure period of 9 days*

Conc. pyrethrin I %	Regression coeff.
0·8	11·33
0·7	15·13
0·6	8·40
0·5	13·47
0·4	12·40

lines increased as the concentration decreased, so that the lines crossed each other in the region of 6·5 probits (93% kill). The results of the 6th day count were more erratic.

It was decided to count dead beetles only in future experiments, because (*a*) the experiments with Nylon showed that some badly affected beetles might recover, (*b*) the distinction of badly affected from slightly affected beetles allows more bias in counting than separation of those dead from the remainder, and (*c*) at the higher concentrations of pyrethrum very small deposits and a short exposure period

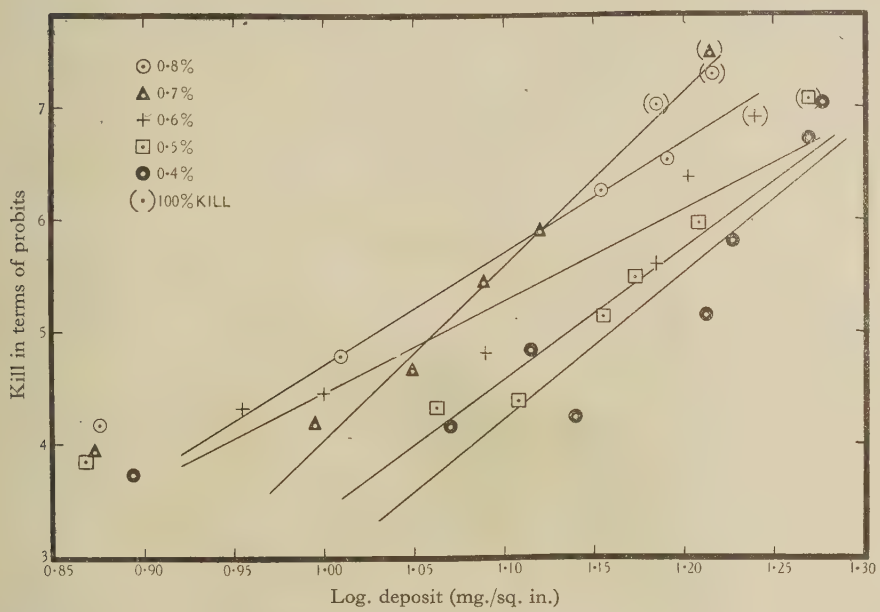

Fig. 3. Provisional regression lines for different concentrations of pyrethrin I in Shell Oil P. 31; data from Exp. 49. *T. castaneum* beetles exposed on Whatman filter paper no. 544 for 9 days. Beetles counted shortly before start of exposure.

There was no correlation between concentration and slope and, as the same toxic principle was used throughout, the differences between the regression coefficients were presumably due to some uncontrolled experimental factor or combination of factors.

Similar sets of data were obtained after exposures of 3 and 6 days. The results were more heterogeneous after 3 days' than after 6 or 9 days' exposure, when they were about equal. The slopes of the regression lines for each concentration tended to decrease as the period of exposure lengthened.

An analysis was made of the numbers of beetles dead plus badly affected after 3 and 6 days: the totals were too high by the 9th day for satisfactory statistical treatment. The data of the first count were more homogeneous than the corresponding data for dead beetles only, but the slopes of the regression are necessary to give a reasonable range of mortalities.

The results of this preliminary experiment suggested that further trial of no. 544 filter paper was warranted. It was assumed that, by the use of filter paper, much of the variation in the greaseproof-paper experiments attributed to inequalities in absorption of insecticide had been overcome, resulting in the observed increase in homogeneity of the results.

The differences in slopes of the regression lines at each period of exposure were interpreted as differences in the resistance of the insects greater than might be expected through sampling error, and it was thought that a probable contributory cause was the violent disturbance of the beetles during counting with a sucking tube shortly before the start of the exposure period. This idea was tested experimentally.

B. *Subsequent experiments (nos. 51–61)*

The investigation was continued to improve the method used in the preliminary experiment and to ascertain the lower concentration which could just be reliably distinguished from the standard insecticide containing 0·8% pyrethrin I in Shell Oil P. 31. In view of the break in the slope of the regression line in the preliminary experiment and because very high percentage kills do not give a valid contribution to the χ^2 test, the range of mortalities for statistical analysis was limited to 30–90% (20–90% in Exp. 51) and the deposits chosen accordingly. The experiments are summarized under headings indicating the factors under consideration.

(1) *The effect of disturbance on the insects*

A test on a scale similar to the preliminary experiment was undertaken using concentrations of 0·8, 0·7, 0·6, 0·4 and 0·325% pyrethrin I. The beetles, after removal from the subculture jars, were allowed

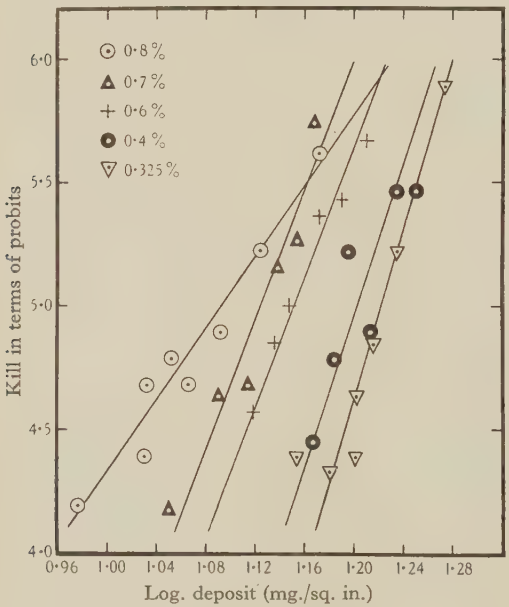

Fig. 4. Provisional regression lines for different concentrations of pyrethrin I in Shell Oil P. 31; data from Exp. 51. *T. castaneum* beetles exposed on Whatman filter paper no. 544 for 6 days. Beetles counted 18 hr. before start of exposure.

to mix for 2–3 hr. at 24° C. and 70% R.H. in a 7 lb. jar containing crumpled paper and were then transferred in batches of fifty to the 4 oz. jars, in which they were starved overnight under the same conditions of temperature and humidity. In this way, beetles had at least 18 hr. in which to recover from the effects of the counting process. To place the insects on the sprayed papers, their foothold was loosened with the aid of a camel-hair brush and they were tipped gently into a ring of thin glass, 2 cm. high and 4 cm. diam. When all had been confined in the ring, it was quickly replaced by a funnel.

A second change introduced in this experiment was the inclusion in the analysis of only those mortalities between the limits of 20 and 90% inclusive.

The result of these changes is shown in Fig. 4 in which the provisional regression lines are drawn for the data of the count of percentage mortality after 6 days. The fit of the points to the provisional regression lines after 6 and 9 days' exposure is shown in Table 4.

TABLE 4. *Exp.* 51. *Fit of points to regression lines*

Exposure days	Conc. pyrethrin I %	χ^2	n	P (approx.)
6	0·8	2·16	6	0·90
	0·7	2·83	4	0·60
	0·6	0·52	4	0·97
	0·4	5·24	4	0·25
	0·325	6·94	5	0·23
9	0·8	3·21	3	0·38
	0·7	0·36	5	0·99
	0·6	2·14	7	0·95
	0·4	8·59	5	0·14
	0·325	4·36	4	0·38

The added values of χ^2 and n for the two periods of exposure gave the reasonable values $P=0{\cdot}76$ and 0·77 (approx.) respectively.

The slopes of the regression lines are given in Table 5.

TABLE 5. *Exp.* 51. *Slopes of the regression lines*

Exposure days	Conc. pyrethrin I %	Regression coeff.
6	0·8	7·17
	0·7	12·40
	0·6	11·83
	0·4	11·02
	0·325	14·35
9	0·8	6·99
	0·7	7·25
	0·6	9·24
	0·4	9·63
	0·325	12·19

The regression lines tended towards parallelism at both periods of exposure with one exception in each set. On the whole the slopes showed much greater uniformity than in the preliminary experiment.

It was concluded that it would be advantageous, before exposure to the insecticide, to allow the beetles at least 18 hr. in which to recover from the disturbance of the counting process.

A confirmatory experiment (no. 52) was made. A well-mixed population of beetles was divided into two similar lots, one being counted into batches of fifty and allowed to remain undisturbed for 18 hr. before exposure and the other being counted just before exposure. The numbers dead were recorded

after 6 and 9 days on similar ranges of deposits of 0·8% pyrethrin I in oil. The results showed that, when the beetles were allowed to recover from the disturbance caused by counting, the observed mortalities formed a more homogeneous series. In all later experiments the beetles were randomized and counted into batches of fifty on the day before the test.

(2) *Classification of beetles after exposure*

In preliminary tests with Nylon some beetles classified as badly affected after exposure for 6 days recovered sufficiently by the 9th day to be classified as slightly affected or even as normal. Although partial or complete recovery was not noted in the experiments on filter paper no. 544, an investigation was made of the behaviour of beetles, exposed to a pyrethrum film until badly affected, and then removed to wholemeal flour in which they might feed and breed.

Five batches of fifty *T. castaneum* beetles were exposed to 10–11 mg./sq. in. of 0·8% pyrethrin I on no. 544 papers. Two batches remained on the papers for the full 9 days' observation period by which time the kills were 62 and 68%. The other three batches were removed from the films after 3 days' exposure, when about 96% were badly affected and 4% dead, and placed in separate jars of flour. After 5 weeks at 24° C. and 70% R.H. the insects were sieved from the flour. About 56% of the beetles survived and the cultures contained many larvae. Thus, the 'badly affected' grade contained too wide a range of physiological states to be a satisfactory measure of insecticidal efficiency.

(3) *Effect of oil alone*

Rather high deposits of the lower concentrations of pyrethrum were required to cover the desired range of mortalities, and the quantity of carrier oil applied might have influenced the number of deaths recorded. Beetles, in batches of fifty, were therefore exposed to films of oil alone and were classified and counted after 6 and 9 days (see Table 6).

Deposits up to 20 mg./sq. in. caused few deaths but beetles may be slightly affected in 6 days by deposits exceeding 15 mg./sq. in., and in 9 days by more than 6 mg./sq. in. The effect of the quantity of oil alone would be unimportant, therefore, provided that experimental results were based on kill only and deposits did not exceed 20 mg./sq. in.

(4) *Period of exposure*

In the preliminary experiment beetles were counted after exposure to the films for 3, 6 and 9 days, and a wide range of deposits was required to get a suitable range of kills at each examination. Since each batch of insects could not be examined after exactly 72, 144 and 216 hr., the count at 3 days, when the errors in the period of exposure would be greatest, was omitted.

TABLE 6. *Exp.* 53. *Percentages of dead and affected beetles after exposure to various deposits of Shell Oil P.* 31

	6 days			9 days		
Deposit mg./sq. in.	Dead	Badly affected	Slightly affected	Dead	Badly affected	Slightly affected
2·14	8	0	9	8	0	0
5·21	4	0	0	8	0	2
8·10	2	0	0	8	0	12
10·46	0	0	0	6	2	22
13·00	4	2	0	6	0	26
14·14	8	0	0	10	0	20
15·03	6	2	0	14	2	28
15·74	0	0	4	4	0	44
16·34	4	2	10	12	0	36
17·14	2	0	18	8	8	32
18·43	2	2	16	4	6	46
19·96	2	2	24	14	8	30
26·34	44	6	50	60	10	30
Control 1	4	0	0	6	0	0
2	0	0	0	2	0	0
3	2	0	0	4	0	0
4	4	0	0	4	0	0

Table 7 summarizes data relating to homogeneity of results and slopes of regression lines for the 6 and 9 days' counts of undisturbed insects in all tests using 0·8% pyrethrin I on Whatman filter paper no. 544.

The reduction in value of P between the 6th and 9th day counts of Exp. 51 was due to one aberrant point at the second count. The two low values of P in Exp. 55 cannot at present be explained in view of the general tendency for P to be consistently high at the longer period of exposure. This consistency indicates that some factor may have been introduced into the technique which influenced the normal sampling error. A series of high values of P does not invalidate the test, but more experimental work is needed before an explanation can be given for this unexpected tendency. In general, a satisfactory

degree of homogeneity was achieved in the data at both periods of exposure.

The regression coefficients showed considerable variation after 6 days', but were more uniform after 9 days' exposure. Comparison by a '*t*' test of each coefficient with the mean of the seven values for the appropriate period of exposure showed that the figures for the 6 days' exposure in Exps. 51 and 61 were outside the expected range. Combination of the probabilities from this test of significance to give χ^2 for each period of exposure (see Fisher, 1934, § 21.1) showed that the values of the 9 days' count fell into a homogeneous series, whereas those of the 6 days' count were heterogeneous. The data of Table 7, together with those from exposure of beetles to lower concentrations of pyrethrum, indicated that the regression lines were normally less

TABLE 7. *Homogeneity of data and slopes of regression lines for* 0·8% *pyrethrin I with exposure periods of* 6 *and* 9 *days*

	6 days		9 days	
Exp.	*P* (approx.) from χ^2 test	Regression coeff.	*P* (approx.) from χ^2 test	Regression coeff.
51	0·90	7·17	0·38	6·99
52	0·90	9·07	0·86	5·28
55	0·04	16·47	0·08	8·37
56	0·50	12·78	0·86	5·32
59	0·58	8·77	0·79	9·70
60	0·66	14·79	0·95	7·82
61	0·99	5·98	0·98	6·25

steep after the longer period. A low regression coefficient indicates a greater range of resistance of the population from which the samples of insects are drawn but is advantageous in that a wider range of deposits may be employed to cover a given range of mortalities. Although more uniform results were obtained with the longer exposure period, it is inconvenient to maintain an experiment for so long, because of the space required in the controlled temperature and humidity room. The best point at which to balance the opposing factors can be decided only by further experiment, e.g. by trial of periods of exposure between 6 and 9 days.

(5) *Variation in resistance of the standard insects*

Although an attempt was made to breed a reasonably uniform stock of *T. castaneum* beetles by the application of standard cultural methods, some variation in the general level of resistance of samples of the insects drawn from the cultures at different times is bound to occur; time was not available to ascertain whether this variation could be minimized by the use of beetles bred from a single original pair. On one occasion when the supply of adults was less than the demand, the number of main cultures was increased by addition of beetles from the general insectary stock. A bacterial disease was thus introduced and, although all cultures seen to be affected were immediately destroyed, the disease was not completely eliminated. Some of the differences in susceptibility of the insects used in different experiments may be attributable to this cause. These differences are evident both in the slopes of the lines representing exposure to 0·8% pyrethrin I, as shown by the regression coefficients in Table 7, and in changes in the positions of the dosage-mortality curves illustrated in Table 8 by the deposits of 0·8% concentration required to effect a 50% kill of the population. These deposits were estimated from the regression equations derived from the transformed experimental data.

The figures in Tables 7 and 8 suggested that, although the mean susceptibility of the beetles in different cultures did not vary much, there were

TABLE 8. *Deposits of* 0·8% *pyrethrin I required to kill* 50% *of* Tribolium castaneum *beetles drawn from different main cultures*

	Median lethal deposit (mg./sq. in.)	
Exp.	6 days	9 days
51	11·0	10·2
52	10·8	9·9
55	11·5	10·5
56	10·6	10·2
59	10·4	9·9
60	11·2	10·3
61	10·7	10·6

appreciable differences in the ranges of susceptibility of different cultures. Care must be taken, when two or more main cultures are mixed, that the beetles are adequately randomized before being counted into batches.

(6) *Limits of the test*

Information on this point was gathered from experiments in which different concentrations of pyrethrum were compared with a standard containing 0·8% pyrethrin I.

In Exps. 51, 56 and 60 concentrations of 0·8, 0·7 and 0·6% pyrethrin I were compared and in each instance 0·6% could be distinguished with confidence from 0·8% by visual examination of the graphs of the transformed dosage-mortality data, since there was no overlapping of the points representing the two concentrations after either 6 or 9 days' exposure.

The separation of 0·7 from 0·8% can be achieved with fair, but not complete, certainty: see Table 9, in which the positions of the regression lines are compared by the direct χ^2 test (Bliss, 1935*b*).

The values for *P* show that a concentration of 0·7% was distinguished from 0·8% in two of the three experiments. In no. 60 the test failed to separate the two concentrations at the first count, but succeeded at the second. The regression lines of

Exp. 60 for an exposure of 9 days to 0·8, 0·7 and 0·6% pyrethrin I are shown in Fig. 5.

Two tests were made to compare 0·65% pyrethrin I with the standard. After 6 days' exposure there was a satisfactory degree of distinction between

TABLE 9. *Comparison of positions of regression lines for 0·8 and 0·7% pyrethrin I*

Exp.	Exposure days	χ^2	n	P (approx.)
51	6	6·39	1	0·012
51	9	7·31	1	0·008
56	6	39·76	1	<0·001
56	9	9·66	1	0·003
60	6	2·15	1	0·15
60	9	11·33	1	<0·001

the regression lines for the two concentrations, but they only just agreed for parallelism. At the 9th day count the lines were parallel and completely distinct as the points did not overlap in either experiment.

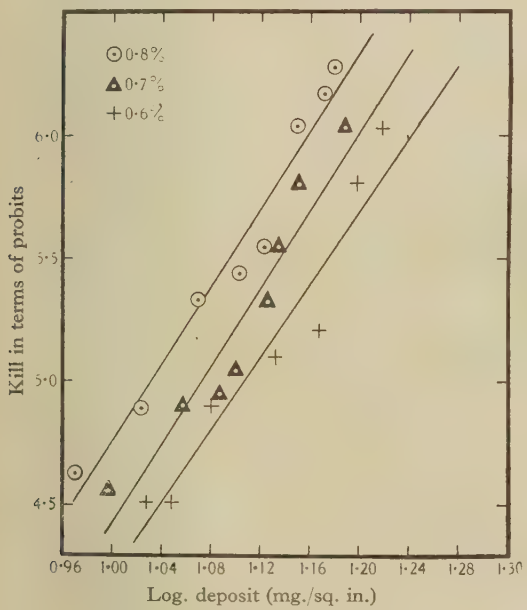

Fig. 5. Provisional regression lines for comparison of toxicities of three concentrations of pyrethrin I in Shell Oil P. 31; data from Exp. 60. *T. castaneum* beetles exposed on Whatman filter paper no. 544 for 9 days.

The present technique will therefore distinguish reliably 0·65 from 0·8% pyrethrin I, and the limit can probably be raised to 0·7%, when more knowledge has been gained of the types and extent of variation which may occur and the underlying causes.

(7) *The confinement of beetles under funnels*

The experiments with Nylon showed that *T. castaneum* beetles, when irritated by pyrethrum, secrete a substance which gives a pink reaction with Nylon. This substance was found by Roth & Howland (1941) to be injurious to *Tribolium* beetles. Although the toxic vapour was probably removed from the atmosphere under the funnels by solution in the oil of the film, other methods of confining the beetles during exposure to the insecticide were considered. Moreover, one of the chief reasons for devising a film test was to be able to detect and measure the changes in toxicity of pyrethrum-in-oil solutions on addition of certain chemicals intended to increase the insecticidal efficiency. Some of these activators may be slightly volatile and it is necessary to avoid building up a toxic concentration of vapour under the funnels. The use of funnels 6 cm. diam. and glass rings, 6 cm. in diam. and 2 cm. deep, was compared. Two series of Whatman filter papers no. 544

TABLE 10. *Statistical data on comparison of funnels and rings for confining beetles on insecticidal films*

Container	Exposure days	χ^2 for fit of points to regression lines	n	P (approx.)	Regression coeff.
Funnels	6	2·04	3	0·58	8·77
	9	1·03	3	0·79	9·70
Rings	6	1·00	3	0·80	7·50
	9	1·09	4	0·90	5·97

were sprayed with approximately equal ranges of deposits of 0·8% pyrethrin I and beetles from a randomized population were exposed under funnels on one series of papers, and under rings on the other. The homogeneity of the data was not affected by the use of rings: see Table 10, in which are included the coefficients indicating the slopes of the regression lines.

The lines for funnels and rings were in good agreement for parallelism at 6 days, but were more divergent at 9 days, when that for the funnels was unexpectedly steep. The use of glass rings instead of funnels does not alter the validity of the technique. The deposits required to achieve a 50% kill at the two periods of exposure were computed from the regression equations (Table 11).

TABLE 11. *Median lethal deposits of 0·8% pyrethrin I on Whatman filter paper no. 544*

Exposure days	Container	M.L.D. mg./sq. in.
6	Funnels	11·0
6	Rings	12·0
9	Funnels	9·8
9	Rings	10·1

For a 6-day period of exposure, the extra amount of insecticide which had to be applied for a 50% kill, if rings were used instead of funnels, was only 1 mg./sq. in. The difference in the figures for the 9-day count was not so reliable, owing to the departure from approximate parallelism.

THE TECHNIQUE OF A COMPARATIVE FILM TEST

On the basis of the experimental work, it is possible to describe a technique by which pyrethrum preparations, intended for use against certain insect pests of stored products, may be compared by their film effect for insecticidal efficiency with a standard containing 0·8% (w/v) of pyrethrin I in Shell Oil P. 31 and the limits of the test fairly closely defined.

A. *Description of the technique*

The following description of the method differs in one or two minor particulars from that used during the experimental work, chiefly because a new type of spray tower is available (see Potter, 1941).

Method for breeding the standard insects

The standard insects are flour beetles, *Tribolium castaneum* Herbst. The main cultures, in which insects are bred, and the subcultures, in which beetles mature, are maintained at 24° C. and 70% R.H. The culture jars are systematically numbered and stand in oil on metal trays, to avoid infestation by mites.

(*a*) *Main cultures.* Wholemeal flour, made from North Manitoba no. 1 wheat, is sterilized by heat and passed through a 24-mesh sieve. The flour should then preferably be conditioned at the desired temperature and relative humidity before use. Sufficient to fill a 1 lb. jar to the base of the neck (180–190 g.) is put into a 2 lb. jar, 100 beetles, 0–2 weeks old, are added and the jar is covered with muslin. A set of main cultures is started each week. After 8 weeks the beetles are removed by means of an 18-mesh sieve and destroyed. The newly emerged adults are then sieved off at intervals of 14 days.

For starting new main cultures, beetles are used immediately after their removal from the flour and the origin of the parent beetles is recorded. The remaining beetles, removed from culture on the same day, are put in a 7 lb. jar, containing crumpled paper, and stored at 24° C. and 70% R.H. until the following day, when they are transferred to the subculture jars. Although by this method the relationship between main and subcultures cannot be traced in detail, better randomization of stocks is ensured and all subcultures used during any 1 week period should be comparable.

(*b*) *Subcultures.* The young beetles 0–2 weeks old, which have been left overnight in a 7 lb. jar, are counted into batches of 500. Each batch is transferred to a 1 lb. jar half full (100 g.) of sieved and sterilized wholemeal flour. Beetles are kept in these jars under the standard conditions for 3 weeks, so that at the time of testing they are 3–5 weeks old.

(*c*) *Beetles for tests.* At least 10% more beetles than will be required are sieved from subcultures on the day before exposure to the insecticide. They are placed in a 7 lb. jar, containing crumpled paper, and left to mix for about 3 hr. They are then removed from the paper and counted into random batches of fifty, which are put in 4 oz. jars and left in the constant-temperature and humidity room overnight. Each jar is closed with a perforated muslin-covered screw cap and contains, as a foothold, a strip of index card, $3 \times \frac{3}{4}$ in., bent at an acute angle on the short axis. At least 1 hr. before the beginning of the exposure period, beetles clinging to the strips are gently brushed off and the card removed from each jar.

Preparation of films

(*a*) *Apparatus.* Some form of spraying tower designed to give a fairly uniform distribution of insecticide over an area about 9 cm. diam. is necessary: that described by Potter (1941) is recommended.

The filter paper, on which the film of insecticide is to be deposited, is supported upon the points of 8–10 evenly spaced drawing pins pressed through a disk of cardboard centred by the adjustable grips of the spray table. The disk has a diameter 2–3 mm. greater than that of the filter paper.

(*b*) *Deposition of films.* A small hole is punched near the edge of a 7 or 9 cm. circle of Whatman filter paper no. 544, so that it can be hung for weighing on a wire hook on one arm of a balance. Alternatively, if the balance pan is large enough, the paper may be supported by pins through cardboard as on the spray table. The filter papers are stored in a dust-proof cover in the laboratory to maintain equilibrium with local conditions of relative humidity and are handled with forceps.

A filter paper is weighed, laid on the points of the support and the whole lowered carefully between the grips of the spray table. The table is raised to the spraying position and the air supply to the nozzle turned on. When the mercury in the manometer has reached the desired steady level (the equivalent of 15 cm. of free mercury is satisfactory), a measured amount of insecticide is delivered from a hypodermic syringe into the reservoir attached to the nozzle. During spraying a watch is kept on the manometer reading, and the time of spraying is noted as a check on blocking of the nozzle. When the charge of insecticide has been used, the air is shut off and the paper immediately removed and weighed, so that the mean deposit per unit area may be known accurately. The nozzle is cleaned by spraying 1 ml. of carbon tetrachloride and continuing the passage of air for 1 min. or so after the liquid has gone through.

(*c*) *Insecticide.* The insecticide used up to the present as the standard, contained 0·8% pyrethrin I (w/v) in Shell Oil P. 31. It was made by dilution with the oil of a specially prepared commercial concentrate, stabilized on receipt at the Laboratory by the addition of 2·5% by volume of a 10% solution (w/v) of pyrocatechol in ether. The concentrate was assayed chemically for pyrethrin I content at the Imperial Institute. Other concentrations were prepared as required for comparison with the standard and all experimental work was limited to comparison

of straight pyrethrum solutions. Different samples of concentrate varied noticeably in colour and quantity of sediment and a better standard than one based on pyrethrin I content alone is badly needed.

(*d*) *Range of deposits.* A range of about nine deposits is recommended, in order to cover mortalities of 30–90% at both 6 and 9 days' exposure. With the *T. castaneum* stocks in use at this Laboratory the required deposits of 0·8% pyrethrin I are about 10–17 mg./sq. in. (1·55–2·62 mg./sq. cm.). The whole range should be covered in 6 or 7 steps and 2–3 additional deposits made near 13 mg./sq. in. (2·00 mg./sq. cm.) to fix the position of the regression line in the region of 50% kill with more certainty. For a concentration of 0·6%, the range of deposits should be about 11–18 mg./sq. in. (1·71–2·79 mg./sq. cm.).

The volume of insecticide needed for a given deposit may be read from a calibration curve appropriate to the temperature during spraying, or may be determined by trial based on previous experience. With an insecticide of unknown strength a preliminary experiment must be undertaken to ascertain a suitable set of deposits, or a range about twice that of the standard should be covered in double the number of steps.

(*e*) *Exposure of beetles.* After spraying, each filter paper is laid on a glass plate and a glass ring, 6 cm. diam., 2 cm. high and with the edges ground flat, is stood upon it. A batch of test insects is gently tipped from a 4 oz. jar into each ring: beetles clinging to the jar are detached with a soft camel-hair brush. Control beetles are confined upon untreated filter papers. The glass plates, each usually supporting 4–6 sets of papers, rings and insects, are kept at 24° C. and 70% R.H. for the duration of the observation period. The major part of the exposure should take place in the dark.

(*f*) *Observation of results.* Until more data are available for forming an opinion whether a single count can be relied upon, it is recommended that the beetles should be examined 6 and 9 days after the start of exposure. The beetles are observed *in situ* on the papers under a low-powered binocular microscope. Dead insects, i.e. those showing no spontaneous movement even when lightly pressed on the metasternum, are removed and their number noted. Living beetles are disturbed as little as possible.

(*g*) *Precautions to be observed.* Glass jars, plates and rings must be scrupulously clean and free from traces of alkali, which is liable to remain on the surface after washing in soapy water. All glass apparatus must be protected from drifting spray mist and particular care taken to ensure that the mist cannot enter jars containing beetles awaiting exposure on the films.

B. *Analysis of results*

Comparison of insecticides should be made on the results of the 9 days' count, unless there is reason to suppose that the figures for 6 days would be more reliable. The data, representing observed mortalities between 30 and 90% for known deposits of insecticide, are analysed statistically by Bliss's method (1935 *a*, *b*). Data obtained from a film test, as described in this paper, should be homogeneous with respect to goodness of fit to the provisional regression line. When two insecticides are being compared, agreement in position and slope of their regression lines should be compared by the direct χ^2 test (Bliss, 1935*b*). If the data are heterogeneous and it is desired to proceed with the comparison, the '*t*' test must be used.

C. *Limits of the comparative test*

Experiments showed that 0·65% pyrethrin I in Shell Oil P. 31 can be clearly distinguished from 0·8%. In most instances the test will distinguish 0·7 from 0·8%, and 0·7% should ultimately become the reliable limit. Such a limit is considered satisfactory in relation to the intended use of this film test.

The work was confined to comparison of straight pyrethrum solutions in heavy oil and no attempt was made to evaluate insecticides containing activators. This must be done before the test can be employed with confidence for deciding whether or not a commercial preparation is equivalent in toxicity to the standard 0·8% pyrethrin I solution.

D. *Comparison with the Peet-Grady method*

The method most widely used for comparing insecticidal preparations of the pyrethrum type with a standard is the Peet-Grady method for evaluating liquid household insecticides (Anon. 1942). The test was designed for preparations of small pyrethrin content (about 0·1% total) in volatile oils of low viscosity. The standard is a pyrethrum preparation in a refined kerosene oil, compounded and distributed in the U.S.A. under the name 'Official Test Insecticide' by the National Association of Insecticide and Disinfectant Manufacturers. The standard insect is the housefly, *Musca domestica* L., reared under controlled conditions and of known age.

The method is based upon the replication of a fixed dose of insecticide, chosen so that the official preparation causes a mortality of 30–55% among flies of suitable resistance. The regression lines are not computed. A check is kept upon variation in the results by the proviso that in the large group method the mortalities in replicated tests with the official test insecticide shall agree within 10%, and in the small group method the standard error of the mean difference in kill of the two insecticides must be less than 3.

In comparison, the film technique described in this paper was devised for evaluating insecticides with a high concentration of toxic principle, e.g. 1·6% total pyrethrins, in a non-volatile oil of high viscosity. The standard insecticide was made by

dilution with the oil of a commercial concentrate, the pyrethrin I content of which was determined chemically. This standard, containing 0·8% pyrethrin I (w/v), is tentative.

When the technique has been tried in other laboratories and more is known of the variations in resistance of stock insects, it should be feasible to prescribe limits within which the resistance of the population used in any test would be acceptable.

The method now described involves the computation of regression lines by a method of statistical analysis requiring the use of a calculating machine. Within limits a comparison of the toxicity of two insecticides can be made by visual appraisal of the extent of overlapping of the transformed dosage-mortality data, when plotted graphically for drawing the provisional regression lines. The technique could be simplified to one similar to the Peet-Grady method, i.e. replication for all materials under test of a deposit giving about 50% kill with the standard and comparison of the mean mortalities observed in batches of beetles after exposure. Comparison of mortalities at a fixed dose is valid only if the regression lines are approximately parallel. Until information is available upon the slopes of regression lines representing the effects of insecticides containing pyrethrum and/or other toxic substances, the reduction of the technique to a simpler form for general use is not advisable, particularly as much useful experimental information would be lost by the change.

This work was carried out as part of the research programme of the Pest Infestation Laboratory and is published with the approval of the Department of Scientific and Industrial Research.

Information on the progress of the investigation was exchanged from time to time with Dr F. Tattersfield, Dr J. T. Martin and Dr C. Potter, who were working at Rothamsted Experimental Station upon the production of a pyrethrum-oil extract of accurately defined specification for use as the standard in this type of biological evaluation.

The senior author is grateful for the encouragement given during consultations with Prof. J. W. Munro and Prof. D. Keilin. Valuable discussions on the statistical analysis and interpretation of the experimental data were held with Mr E. D. van Rest, during the course of which he gave much helpful advice.

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The preparation of a standard pyrethrum extract in heavy mineral oil, with observations on the relative toxicities of the pyrethrins in oil and aqueous media

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(With 1 Text-figure)

The preparation of a pyrethrum extract in highly refined heavy mineral oil, suitable for use as a basis of reference in the biological evaluation of commercial pyrethrum-heavy oil preparations, is described. The solution was standardized with respect to colour, resin content and equal proportions of the pyrethrins. A standard solution containing pyrocatechol remained stable, when judged by the chemical determination of the pyrethrins, over a period of months.

When applied in a heavy mineral oil medium, pyrethrin II was shown to possess a toxicity to *Tribolium castaneum* Hbst. approaching, if not equal to, that of pyrethrin I. When applied in an aqueous medium the toxicity of pyrethrin II was very much lower than that of pyrethrin I.

Introduction

The rapidly increasing use of pyrethrum extracts in heavy mineral oil in the control of insect pests calls for methods of determining their insecticidal efficiencies. In view of the possibility of the addition of other substances to commercial preparations, the chemical determination of the pyrethrins cannot be relied upon to assess insecticidal potency. This may only be done satisfactorily by biological tests upon suitable insects.

The change, from time to time, in the resistance of the test insects, even when these are cultured under uniform environmental conditions, raises a difficulty which may be overcome by the incorporation in the toxicity tests of a standard insecticide, to which the potencies of the solutions under test may be related. The theory underlying the comparison of the toxicities of the standard insecticide and the test solutions was outlined by Bliss (1939).

To make comparison effective the standard insecticide should exhibit the same type of physiological action as the test solution. The action of pyrethrum is unique among contact insecticides in its rapidity and manifestations, and the choice of the standard insecticide is limited at present to a pyrethrum preparation. Benzophenone, suggested by Gnadinger (1936, p. 102) as a standard insecticide in the evaluation of fly-sprays, has been replaced by pyrethrum extract in light mineral oil (Simanton, 1937; Campbell, 1938).

The National Association of Insecticide and Disinfectant Manufacturers Inc., New York, have, since 1936, supplied to manufacturers a standard pyrethrum insecticide for use in the evaluation of fly-sprays. I am indebted to the Secretary of the Association for permission to give details of its nature. The official test insecticide (O.T.I.) is prepared from a mixture of pyrethrum concentrate extracts supplied by several manufacturers. Four laboratories determine the pyrethrin content of the mixture of concentrates, and dilution is made with a deodorized kerosene of approximately 0·775 sp. gr., and boiling range of approximately 380–520° F., to give a total pyrethrin content of 0·1%. The diluted product is again checked chemically by the mercury reduction and Seil methods, the former giving results approximately 10% lower than the latter. The O.T.I. contains no added materials for stabilization or other purposes, but consists only of the extractives from the pyrethrum and the oil carrier. The official test insecticide has proved of great value in the evaluation of fly-sprays, and its introduction marked an important step forward in the biological testing of such insecticides.

During 1940 and 1941, this Department was concerned in the biological evaluation of pyrethrum extracts in heavy mineral oil containing much higher quantities of the pyrethrins than those present in the O.T.I. (Parkin & Green, 1943; Tattersfield & Potter, 1943). The need for a suitable standard insecticide became apparent and its preparation was undertaken.

In addition, information was needed on the relative importance in causing mortality of the test insects of the two pyrethrins in heavy oil solution. Recent work showed that, in kerosene solution, pyrethrin I tends to produce a higher mortality than pyrethrin II. Haller & Sullivan (1938) and Sullivan *et al.* (1938) found a pyrethrin I concentrate in kerosene to be more toxic to houseflies than a pyrethrin II concentrate. McGovran *et al.* (1941) found that a kerosene solution rich in pyrethrin I caused higher mortalities of the American cockroach than

a pyrethrin II concentrate in the range of 17–77% kill, but that at the 80–83% mortality level the effects of the pyrethrins were approximately equal.

The relative toxicities to adult *Tribolium castaneum* Hbst. of concentrates of the pyrethrins in heavy mineral oil were therefore determined, together with their relative effects upon the same insect when applied in an aqueous medium. The possibility remains of synergistic action between the pyrethrins. Ripert & Gaudin (1935) found that on injection into frogs, a mixture of the pyrethrins was more toxic than the pyrethrins administered separately.

Potter's apparatus (1941) was used for the biological trials. Mortality was assessed by the summation of the percentages of badly affected, moribund and dead insects. Standard errors were calculated on the percentage mortalities before allowing for the controls.

Experimental

The final standard solution used as the basis of reference in the biological evaluation of heavy oil preparations should meet the following requirements:

(1) It should contain the true pyrethrins I and II in a definite proportion.

(2) It should contain a definite amount of pyrethrum oleoresin.

(3) It should not undergo more than slight deterioration on storage.

(4) It should be possible to check the pyrethrin content by chemical means.

(5) It should be reproducible.

(1) *The definite proportion of the true pyrethrins.* Pyrethrum flowers vary considerably in the relative proportions of the true pyrethrins present, and the relative proportions found vary slightly according to the methods of analysis used. A 50:50 ratio of the pyrethrins seemed to be most convenient and desirable in the standard oil solution. The methods of obtaining extracts containing the pyrethrins in equal proportion were:

(*a*) To extract flowers which analysis had shown to contain the pyrethrins in equal amounts.

(*b*) To extract selected blends of flowers of known variation of pyrethrin content so that the combined extract would contain the pyrethrins in equal proportions.

(*c*) To treat, by physical means, an extract containing the pyrethrins in unequal amounts so that the contents of I and II would become equalized.

(*d*) To separate an extract of the flowers into fractions containing preponderating amounts of I and II and to add these in calculated quantities to extracts to give equal contents of the pyrethrins.

Methods (*a*) and (*b*) were impracticable as all the flowers available showed an excess of pyrethrin I over II, while with (*c*) much experimental work was needed before a reliable method could be established: method (*d*) was used.

The presence in petroleum-ether extracts of the flowers, and especially in extracts with organic solvents other than petroleum ether, of free acids capable of reacting in the methods of analysis as true pyrethrins, rendered their removal necessary.

(2) *Amount of pyrethrum oleoresin present.* Successive preparations of the standard solution involved the possibility that the amounts of resin extracted from different batches of flowers would vary. Since the resin content of an oil solution might affect the availability of the pyrethrins to the insect or influence the degree of stability of films of the insecticide, the resin content of the oil solution was standardized to $5{\cdot}0 \pm 0{\cdot}5$% by volume.

(3) *The stability of the standard solution.* The value of a standard solution depends to a large extent upon its stability. Tattersfield (1932) and Tattersfield & Martin (1934) showed the value of pyrocatechol and other antioxidants in stabilizing pyrethrum. The addition of an antioxidant was therefore considered, but experiments showed that oil solutions of pyrethrum extract were relatively stable over long periods.

Mixed Kenya and English-grown flowers were extracted with petroleum ether and the extract, made up to a known volume, was analysed with removal of free acids by the Seil (1934) method, using a slight excess of acid in the distillation of the monocarboxylic acid. Aliquots were taken, the solvent was removed, and the resins were dissolved in heavy mineral oil and olive oil to give solutions containing 1·03% by volume of pyrethrin I. After 2 years, during which the solutions were kept mainly in the dark but at intervals in clear-glass bottles exposed to sunlight, the samples were re-analysed. An excess of normal alcoholic potash was ensured during the saponification of the olive oil solution, and the mineral oil was removed by washing with petroleum ether. The monocarboxylic acid in the case of the mineral oil solution was determined by titration as in the Seil method, and with the olive oil solution, to avoid interference, by the mercury reduction method (Wilcoxon, 1936, as modified by Holaday, 1938). In the mineral oil solution the value for pyrethrin I was 0·89% by volume, and with the olive oil solution 0·84%. The deterioration over 2 years was 14–18%.

A third oil solution was prepared from a pale yellow pyrethrum extract, obtained by petroleum-ether extraction of Kenya flowers mixed with decolorizing charcoal. Pyrocatechol (0·25%) was incorporated as a 10% solution in ether. The pyrethrin I content (by calculation from the pyrethrin I content of the extract and checked in the oil solution) was 0·80% by volume. After storage in the dark for 6 months the pyrethrin I content on analysis was 0·81%, no loss of pyrethrin I, detectable by chemical means, occurring over this period.

A fourth solution was prepared by extracting Kenya and Harpenden-grown flowers, mixed with charcoal, with petroleum ether. The pale yellow

extract, made up to volume, contained 0·49% pyrethrin I and 0·43% pyrethrin II by volume. The solvent was removed from an aliquot and the resin was dissolved in heavy mineral oil. Pyrocatechol (0·25%) was incorporated as before. The pyrethrin I content, calculated from the value obtained on the petroleum-ether extract, was 0·86% and the pyrethrin II content 0·74%. On analysis soon after preparation, the solution showed 0·86% pyrethrin I. The solution was kept for 19 months at laboratory temperature in the dark, and re-analysed, when the pyrethrin I content was 0·87%, and the pyrethrin II content 0·71%.

A further solution was prepared by dissolving a petroleum-ether extract of flowers in heavy mineral oil containing a commercially used antioxidant. The pyrethrin I content, calculated from the determined pyrethrin I content of the petroleum-ether extract, was 3·26%. The mean value of four determinations made on the oil solution was 3·16%. After 11 months' storage at laboratory temperatures, the solution was diluted with heavy oil and re-analysed: the pyrethrin I value was 3·06%.

The results of experiments at laboratory temperatures on the stability of pyrethrum-oil solutions, judged on a chemical basis, are summarized in Table 1.

The stability of films. Further investigation was needed before the decision to include or omit an antioxidant in the standard solution could be made. An antioxidant might not be required to prevent deterioration in a bulk oil solution, but there was the possibility that oil preparations exposed as films (as in the biological method of evaluation) would show different rates of inactivation according to whether or not an antioxidant was present. A film stabilized by an antioxidant might then be capable of inducing a higher mortality than a film rapidly deteriorating although of the same initial pyrethrin content. The rate of inactivation of films with and without antioxidants was therefore studied.

Kenya pyrethrum flowers were percolated with acetone, the solvent was removed, and the resin macerated with heavy mineral oil for 2 days. The oil solution was strained through cotton-wool and made up to volume. Analysis showed a content of 1·02% by volume of pyrethrin I. Solutions were prepared containing 0·25% of pyrocatechol and of a commercially used antioxidant added in ether solution, with a control solution containing ether (2·5%) alone. The pyrethrin I content of each final oil solution was 0·72% by volume.

The solutions were sprayed on to circles of Tricoline textile material in Petri dishes. The circles were weighed before and after spraying. Adult *Tribolium castaneum* (four replicates of twenty-five insects each) were confined upon the films with inverted funnels, and the dishes, in randomized positions, exposed in a glasshouse to daylight.

The insects were removed from the films after 24 hr., examined, and fresh insects were placed on the films. These were removed after a further 24 hr., kept at 25° C. and 70% R.H. overnight and examined. A third batch of insects was retained on the films for 18 hr. No intervals elapsed between the removal of the insects from the films and the addition of fresh individuals.

After removal of the third batch, individuals of *Oryzaephilus surinamensis* L. were added, as it was considered likely that the films, while no longer toxic to *Tribolium castaneum* might still affect the more sensitive *Oryzaephilus surinamensis* (see Table 2). There is no indication of a greater retention of activity of the film due to the presence of either antixoidant.

Although little decisive evidence is available to

TABLE 1. *The stability of pyrethrum extracts in oils with and without antioxidants*

Sample	Antioxidant	Exposure	Loss of pyrethrin I %
Pyrethrum extract in heavy mineral oil	None	2 years, mainly in dark	14
Pyrethrum extract in olive oil	None	2 years, mainly in dark	18
Colourless pyrethrum extract in heavy mineral oil	Pyrocatechol	6 months in dark	Nil
Colourless pyrethrum extract in heavy mineral oil	Pyrocatechol	19 months in dark	Nil
Pyrethrum extract in heavy mineral oil	Commercial product	11 months in dark	5

TABLE 2. *The retention of the activity of films with and without antioxidants*

Material	Antioxidant	Film deposit mg./sq. cm.	*T. castaneum* Mortality (%) after 24 hr.	48 hr.	66 hr.	*O. surinamensis* Mortality after 90 hr. %
Pyrethrum extract in heavy mineral oil (0·72% pyrethrin I)	None	2·7–3·0	100	11	3	14
	Pyrocatechol	2·7–2·9	100	7	0	17
	Commercial product	2·7–2·9	100	14	1	14

show the beneficial action of an added antioxidant in delaying loss of activity of pyrethrum-heavy mineral oil preparations (Table 1), the addition of pyrocatechol (in ether solution) to the standard insecticide is advocated. In the biological tests, the ether is likely to be lost from the oil droplets in the process of atomization, but possible changes in spray deposit following the inclusion of ether should be checked by weighing.

(4) *Chemical assay of standard solution.* The pyrethrin content of the standard solution should be checked by the methods now available, on preparation and at intervals, in order to detect possible deterioration.

(5) *Reproducibility of standard solution.* This should be possible at any time and should be independent of the resin and pyrethrin contents of the flowers used. The chemical nature of the resin may vary with flowers of different origin, but this is not considered to be an important factor in the reproducibility of standard solutions.

The preparation of the standard insecticide

It is unlikely that a higher total pyrethrin content than 2% will be used in the biological method of evaluation. This content of total pyrethrins (weight by weight) in the standard solution was decided upon with the possibility of accurate dilution with heavy oil should lower concentrations be required.

Martin (1941) showed that if the ground fully open flowers are mixed with decolorizing charcoal and extracted with petroleum ether the pyrethrins are extracted, but an appreciable proportion of the resin and the pigment are retained by the charcoal. The pyrethrin content of the resin extracted may then be about 40–45%. This method of extraction of the pyrethrins was adopted, the resin content of the standard solution then being brought to a definite and arbitrary level ($5{\cdot}0 \pm 0{\cdot}5$%) by the addition of pyrethrum resin, freed as much as possible from the pyrethrins. All operations were carried out in subdued light.

Preparation of the pyrethrum resin. This was based on the method of La Forge & Haller (1935). Kenya flowers (3 kg.), mixed with decolorizing charcoal (200 g.), were extracted with petroleum ether to give a pale yellow solution. The ratio of pyrethrin I to II in the extract was 1·4:1. The solvent was removed, finally under reduced pressure, and the resin dissolved with warming in glacial acetic acid. An equal volume of acid containing 20% of water was added with swirling, and the solution kept at 0° C. for 2 hr. The precipitated resin was filtered at the pump, washed with cold 90% acetic acid, 80% methyl alcohol and dried in a vacuum desiccator. The final fatty product (fraction A) was pale yellow and lost only 1% of its weight on drying for 45 min. at 100° C. On analysis it contained 3·7% of pyrethrin I (mercury reduction method) and 7·2% of apparent pyrethrin II (Seil method). A further precipitation from acetic acid solution would probably have been of value in reducing these comparatively high contents of occluded pyrethrins.

Preparation of pyrethrin I and II concentrates. This was based on the work of Wilcoxon & Hartzell (1933). The filtrate from the resin precipitation was diluted largely with water and extracted with petroleum ether. The petroleum-ether solution was washed with water and shaken for 30 min. with 80% methyl alcohol, changing the alcohol every 5 min. The methyl-alcohol extracts were combined, diluted largely with water and extracted with petroleum ether. This procedure gave a petroleum ether-soluble fraction of the resin remaining in the filtrate (fraction B) and a methyl alcohol-soluble fraction, also in petroleum ether (fraction C).

The petroleum-ether solutions were washed with water and dried over anhydrous sodium sulphate. The solvent was removed at a low temperature and the products were dissolved in ten times their weights of heavy mineral oil.

The oil solutions were analysed with the removal of free acids. The solution of fraction B contained 3·74% pyrethrin I and 2·09% pyrethrin II, while that of fraction C contained 2·07% pyrethrin I and 5·29% pyrethrin II, by volume. The oil solutions were stored in the refrigerator for use in equalizing the pyrethrin contents in subsequent preparations from different batches of flowers.

Preparation of the final solution. A further extract of 1·5 kg. of flowers mixed with charcoal was made up to a definite volume with petroleum ether and analysed for the contents of resin and the pyrethrins. The resin including the pyrethrins, determined by distillation of the solvent and heating at 90° C. until of constant weight, amounted to 6·37%, while the content of pyrethrin I (mercury reduction) was 1·28% and of pyrethrin II (Seil) 0·91% by volume.

Resin (fraction A, 4·6 g.) in heavy mineral oil and 35 ml. of the oil solution of fraction C were then added to the resin from 305 ml. of the petroleum-ether extract. A little petroleum ether was used to make the additions, the mixture was shaken until all solid material had dissolved and the solution was kept under reduced pressure at a low temperature until no further loss of weight occurred. The solution was then made up to 464 g. (550 ml.) with heavy oil.

The final solution contained 19·4 g. of resin present in the 305 ml. of petroleum-ether extract used, together with 3·5 g. added in fraction C and 4·6 g. added as fraction A. The total amount of resin (27·5 g.) present in the 550 ml. of oil solution was thus equivalent to 5·0% by volume.

The pyrethrin content of the final solution was similarly calculated. 3·90 g. of pyrethrin I and 2·78 g. of II were supplied in the 305 ml. of petroleum-ether extract taken, 0·72 g. of I and 1·85 g. of II were added in the 35 ml. portion of fraction C and 0·17 g. of I and 0·33 g. of II were included in the added

4·6 g. of fraction A. The total amounts of pyrethrin I (4·79 g.) and of pyrethrin II (4·96 g.) present in 464 g. of the final oil solution were thus equivalent to 1·03 and 1·07% by weight respectively.

On analysis the solution showed 1·02% pyrethrin I and 1·17% pyrethrin II.

The addition of the pyrocatechol (0·25%) as a 10% solution in ether was made just before the final addition of the heavy oil.

The value for pyrethrin II (7·2%) obtained in the separated resin (fraction A) was probably an overestimate of the true amount present. The solution from which the resinous material was precipitated contained a greater amount of pyrethrin I than II, and it is unlikely that the resin would occlude so large an excess of pyrethrin II over I. The high value for pyrethrin II in the resin is more likely to be due to interfering acidic material liberated on saponification and titrated as the dicarboxylic acid. If it be assumed that the pyrethrins were retained by the resin in equal proportions, the value for pyrethrin II in the final oil solution is equal to that of pyrethrin I, namely, 1·03% by weight.

A number of standard oil solutions have now been prepared. The intensity of colour, recorded in a Lovibond tintometer, was of the order of 3–4 units of yellow when tested in a layer 1 cm. thick. The incorporation of 0·1 unit of red was needed to obtain an exact match.

Experience in this laboratory has shown that the pyrethrin II values recorded by the Seil method are less satisfactory than the pyrethrin I determinations by the mercury reduction method. Attempts were made, in collaboration with Dr S. H. Harper, to utilize the methoxyl method (Haller & Acree, 1935) for the determination of pyrethrin II in heavy mineral oil solution. The highly refined oil used in preparing the standard solutions did not interfere with the analysis, and values slightly lower than those given by the Seil method were obtained. With extracts of the flowers obtained by the use of organic solvents other than petroleum ether, e.g. acetone and ethylene dichloride, values for pyrethrin II several times higher than those obtained with petroleum-ether extracts resulted. The determination by the methoxyl method of pyrethrin II in commercial pyrethrum-oil preparations was unreliable. The Seil method for pyrethrin II was retained in the preparation and analysis of the standard oil solutions, since this method is likely to be used in the analysis of the commercial products. The standard solutions should be stored preferably in the refrigerator in amber-glass bottles each containing sufficient solution for one test.

The relative toxicities of the pyrethrins in heavy mineral oil and aqueous media

A further quantity of Kenya flowers (1·5 kg.) mixed with charcoal was extracted with petroleum ether to give a pale yellow solution containing the pyrethrins. Fatty material was removed by acetic acid treatment, and the pyrethrins were partially separated between petroleum ether and methyl alcohol as before. The resin soluble in the methyl alcohol was recovered in solution in petroleum ether. The dried petroleum-ether solutions were each made up to 100 ml. and aliquots were analysed for pyrethrin content, with the initial removal of free acidic material. Pyrethrin I was determined by the mercury reduction method and pyrethrin II by the Seil method. In one solution the ratio of pyrethrin I to II was 1·75 and in the other 0·27. The petroleum-ether solutions were stored in the refrigerator.

Aliquots of the solutions were taken as required, the solvent was removed at a low temperature under reduced pressure, and the resins were dissolved in heavy mineral oil or absolute ethyl alcohol. Spraying trials were carried out with as little delay as possible, using adult *T. castaneum* as test insect. The oil solutions were diluted with heavy mineral oil and the alcoholic solutions were added to 0·5% saponin solution, with adjustment of the content of alcohol to 10%. With each experiment, a mixture of the pyrethrin I and II concentrates was included, to gain information on the possibility of synergistic action between the pyrethrins.

For both the oil and water series of tests, insects from cultures (on wholemeal flour) 3 months old were employed. The spray deposits used throughout each series were controlled. The insects were sprayed on Tricoline, and were examined 3 days after spraying with the oil solutions and 2 days after the application of the water sprays (see Tables 3 and 4).

Pyrethrin II values, determined on certain of the spray solutions by the methoxyl method, were 7–12% lower than the values calculated from the pyrethrin II contents of the petroleum-ether solutions, determined by the Seil method.

The logarithms of the concentrations of total pyrethrins are plotted against probits (Bliss, 1935) in Fig. 1A (Exp. 1) and B (Exp. 2). Calculated regression lines are given throughout Fig. 1.

In Exp. 1, the data are closely fitted by one regression line (χ^2, $n=11$, 8·621), the pyrethrin I concentrate, the pyrethrin II concentrate and the mixture showing equal toxicities. In Exp. 2, the data for each preparation were not heterogeneous, neither did the regression lines depart significantly from parallelism, but the pyrethrin I and II concentrates were just significantly different in toxicity. The experiment showed no difference in the toxicities of the pyrethrin II concentrate and the mixture.

It is clear that in the heavy mineral oil medium, the toxicity of pyrethrin II closely approaches that of pyrethrin I. The difference in toxicity between the two concentrates is not sufficiently great for any possible synergistic or antagonistic effect to be detected.

The logarithms of the total pyrethrins are plotted against the probits in Fig. 1C (Exp. 3) and D

(Exp. 4). In both cases, the regression lines for the pyrethrin I and II concentrates differ markedly in position, with the regression lines for the mixtures occupying intermediate positions. In neither case did the three regression lines depart significantly from parallelism. There was a tendency towards heterogeneity throughout the data.

In experiments 3 and 4, the relative toxicities of the pyrethrin I and II concentrates agreed closely with values of 2·60 and 2·69 respectively. In Exp. 3, the mixture of the concentrates showed synergism between the components, the difference between the actual log L.D. 50 and the value predicted by similar action being:

$$Mm = -0{\cdot}178 \pm 0{\cdot}063,$$

in Exp. 4: $Mm = 0{\cdot}045 \pm 0{\cdot}065,$

showing a slight, non-significant antagonistic effect.

TABLE 3. *The relative toxicities to* Tribolium castaneum *of concentrates of pyrethrin I and II and of a mixture in a heavy mineral oil medium*

Conc. (mg./l.) Pyrethrin I	Conc. (mg./l.) Pyrethrin II	No. of insects	Mortality allowing for controls %	S.E. (%) ±
Exp. 1. Deposits 0·7 mg./sq. cm. Threefold replication at each concentration tested				
Pyrethrin I concentrate				
5,000	2,860	62	100	—
2,500	1,430	58	84·5	6·4
1,250	715	56	23·2	10·0
625	358	60	8·3	4·7
Pyrethrin II concentrate				
4,590	16,840	59	100	—
2,295	8,420	59	98·3	2·4
1,148	4,210	62	85·5	2·3
574	2,105	59	44·1	7·2
287	1,052	55	20·0	3·9
Mixture of pyrethrin I and II concentrates				
3,648	5,640	61	100	—
1,824	2,820	62	90·3	4·7
912	1,410	62	40·3	16·2
456	705	61	18·0	11·0
Controls (heavy mineral oil)		64	0	—
Exp. 2. Deposits 0·7 mg./sq. cm. Fourfold replication at each concentration tested				
Pyrethrin I concentrate				
3,162	1,809	83	61·1	6·2
2,000	1,144	78	31·9	13·7
1,408	806	77	14·7	5·3
1,000	572	79	5·0	4·5
Pyrethrin II concentrate				
1,451	5,325	77	68·9	6·9
918	3,368	80	28·4	14·0
646	2,372	85	16·7	8·4
459	1,684	76	13·7	11·2
Mixture of pyrethrin I and II concentrates				
2,307	3,567	79	57·8	11·7
1,459	2,256	80	27·1	8·8
1,027	1,589	80	11·5	5·4
730	1,128	80	7·6	4·5
Controls (heavy mineral oil)		117	4	2·7

TABLE 4. *The relative toxicities to* Tribolium castaneum *of concentrates of pyrethrin I and II and of mixtures in an aqueous medium*

Conc. (mg./l.) Pyrethrin I	Conc. (mg./l.) Pyrethrin II	No. of insects	Mortality allowing for controls %	S.E. (%) ±
Exp. 3. Deposits 9·5 mg./sq. cm. Fivefold replication at each concentration tested				
Pyrethrin I concentrate				
401	229	100	100	—
200	115	103	96·5	2·8
80	46	99	91·4	2·0
40	23	102	52·8	5·9
Pyrethrin II concentrate				
192	702	100	100	—
96	351	99	93·9	2·2
38	140	95	49·3	4·5
19	70	103	33·3	7·8
Mixture of pyrethrin I and II concentrates				
296	466	100	100	—
148	233	103	96·5	2·0
59	93	103	88·2	1·8
30	47	99	52·5	4·6
Controls (alcohol—saponin)		101	17	5·5
Exp. 4. Deposits 9·1 mg./sq. cm. Fivefold replication at each concentration tested				
Pyrethrin I concentrate				
200	115	94	96·8	2·2
100	57	100	73·2	5·7
60	34	107	39·3	4·4
40	23	101	29·6	6·6
20	12	91	7·1	5·0
Pyrethrin II concentrate				
153	561	100	92·9	1·4
77	281	100	58·8	5·1
46	168	105	27·4	3·4
31	112	61*	27·4	6·6
15	56	101	9·2	5·6
Mixture of pyrethrin I and II concentrates				
117	338	97	85·2	1·8
89	169	106	66·0	6·2
53	101	95	29·5	8·0
35	68	89	8·5	3·9
Controls (alcohol—saponin)		99	3·0	2·0

* Remainder of insects of poor quality.

The toxicity of pyrethrin II in the aqueous medium is clearly many times less than that of pyrethrin I, as opposed to its approximately equal effect in the heavy mineral oil medium. The magnitude of the difference in effect of the pyrethrins in aqueous media

is the subject of further work. The results indicate that while pyrethrin I alone is likely to indicate the relative toxicities of extracts of pyrethrum flowers in an aqueous medium (Tattersfield & Martin, 1934) the contents of both pyrethrins need to be taken into account in the assessment of standard insecticide and commercial pyrethrum preparations in heavy mineral oil solution.

a major change in insect resistance in a comparison of Exps. 1 and 4.

The pyrethrin I and II concentrates used in Exps. 1 and 4 were identical in respect of the ratios of the two pyrethrins present. In the pyrethrin I concentrate the ratio of pyrethrin I to II was 1·75 and in the pyrethrin II concentrate 0·27.

The median lethal concentration in the oil Exp. 1

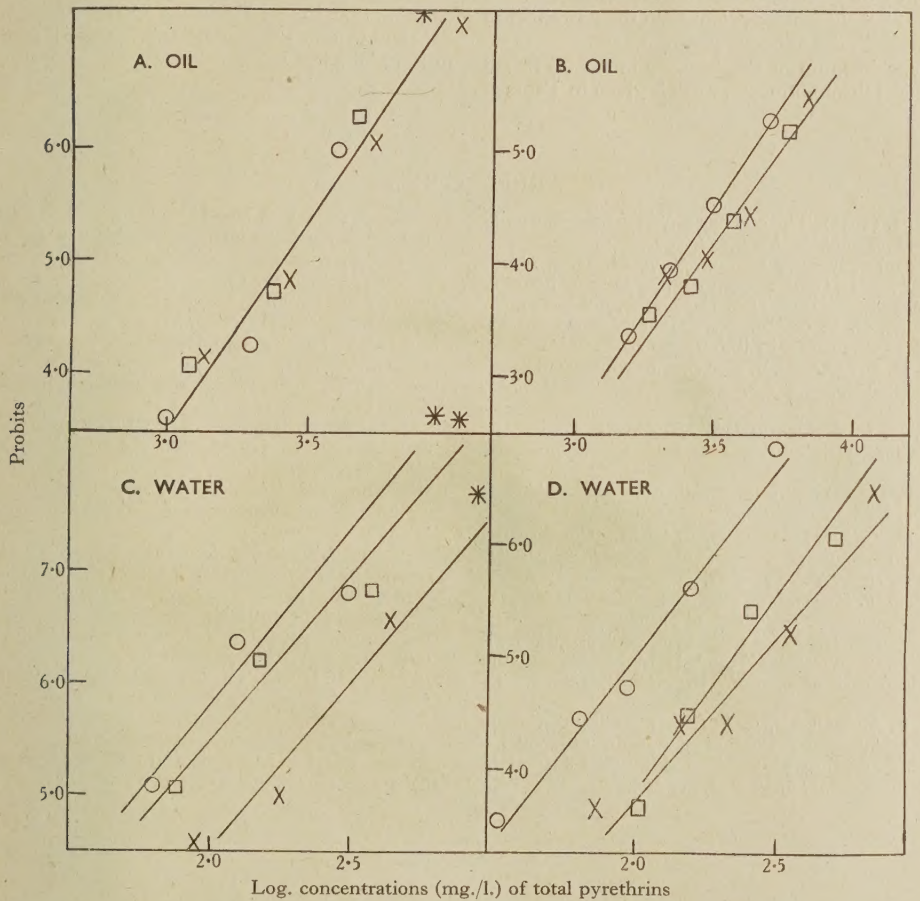

Fig. 1. The relative toxicities of pyrethrin I and II concentrates and of mixtures in heavy mineral oil and aqueous media.

A further point of interest is the relative effectiveness to *T. castaneum* of the pyrethrum preparations when employed in the heavy mineral oil and water media. The insects used in Exps. 1 (oil sprays) and 4 (water sprays) were from the same jars of cultures 3 months old, and were sprayed on Tricoline on successive days. In each case, the treatment of the insects immediately before and after spraying was identical. There can, therefore, be no question of

of both the pyrethrin I and II concentrates was about 2,455 mg./l., and a spray deposit of 0·7 mg./sq. cm. was used. In Exp. 4, using an aqueous medium, the median lethal concentration of the pyrethrin I concentrate was 99 mg./l. and that of the pyrethrin II concentrate 266 mg./l., using in each case a deposit of approximately 9 mg./sq. cm. Assuming the specific gravities of the oil and the alcoholic-saponin solutions to be 0·86 and 1·0 re-

spectively, 0·0020 mg. of total pyrethrins in the case of the pyrethrin concentrates in the oil sprays, 0·0009 mg. in the case of the pyrethrin I concentrate in the aqueous spray and 0·0024 mg. in the case of the pyrethrin II concentrate in the aqueous spray were deposited over 1 sq. cm. at the 50% insect mortality level. The pyrethrin I concentrate in the aqueous spray is thus more efficient than either the pyrethrin I or II concentrates in the oil sprays, but these are slightly more efficient than the pyrethrin II concentrate in the aqueous medium.

The specification of the heavy mineral oil (Shell 24210) used throughout the work is given in Table 5.

TABLE 5. *Specification of heavy mineral oil*

Specific gravity	0·862
Viscosity Redwood 1 at 70° F.	118 sec.
Flashpoint closed	320° F.
Flashpoint open	335° F.
Unsulphonated residue	99·2%

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